User's Manual

Model CSC 5300

Nano-Isothermal
Titration Calorimeter III

Isothermal Titration Calorimeter, User's Manual Revision 40605

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Chapter One: Specifications and Warranty

Calorimeter Specifications

CSC 5300 Nano-Isothermal Titration Calorimeter III Specifications	
Baseline stability	±0.02 µcal/sec/hr
Response Time	15 seconds
Injection interval	150 seconds minimum
Cell volume	1.0 mL (24K Gold)
Precision burette	100 or 250 µL
Volume increment	1–15 µL
Delivery precision	±0.01 µL
Stirring rate	0 to 300 rpm
Temperature Range	2 to 80°C
Power requirements	95-250 Volts, 50-60 Hz.

Table 1: CSC 5300 Isothermal Titration Calorimeter Specifications.

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Chapter Two: Installation

N-ITC III Operating Environment

The best results are achieved with the CSC Model 5300 Isothermal Titration Calorimeter when certain environmental requirements are met. Before beginning the installation of the N-ITC III, the following considerations should be addressed:

System Requirements

This Nano-Isothermal Titration Calorimeter III (N-ITC III) requires an external IBM compatible computer system running Windows 2000 Professional operating system or higher with an available USB port. An optional printer can be attached.

Space Requirements

The N-ITC III requires at least 18” x 18” of bench space. This space should be located near a laboratory bench with sufficient adjacent space to accommodate the computer system and optional printer.

Ambient Temperature Requirements

For optimum performance by the N-ITC III, the ambient room temperature should not deviate by more than ±1˚C, with changes in temperature being gradual shifts instead of fast changes. Depending on analytical requirements, adequate sensitivity may be obtainable with larger ambient temperature fluctuations. Ideally the instrument should not be placed in the direct path of heating or cooling vents, or near windows or doors.

Power Requirements

The Isothermal Titration Calorimeter requires a grounded, single-phase power source. A three-conductor line cord assures a safety ground. The operating voltage and line frequency are set at 95-250 VAC and 50/60 Hz. The N-ITC III and computer system should be plugged into the same surge suppressor. An isolated power line (one which is used only for electrical type instruments with no motors, compressor or heaters) is recommended. Unstable power sources may also require the use of a voltage stabilizer in

order to obtain optimum performance from the N-ITC III.

N-ITC III Setup

The following sequence of steps is followed to prepare the N-ITC III for use:

1. Unpack and inspect the instrument and all components.
2. Place the N-ITC III on a suitable bench.
3. Plug the power cord on the back of the N-ITC III.
4. Setup the computer (and printer if applicable) next to the N-ITC III.
5. Connect the USB cable between the N-ITC III and the computer system.
6. Turn on the computer system.
7. Turn on the power switch located on the back of the N-ITC III.
8. Start the N-ITC III data collection program (ITCRun).

Unpacking and Inspection

Unpack your instrument components as soon as you receive them. Carefully inspect each component for shipping damage. For help in assessing shipping damage or to report any missing or defective parts, contact:

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890 West 410 North, Suite A
Lindon, Utah 84042
(801) 763-1500
(801) 763-1414 FAX
support@calscorp.com (E-mail)
www.calscorp.com (World Wide Web)

Product Items

- Model 5300 Nano-Isothermal Titration Calorimeter III
- User's guide (this manual)
- CSC Proprietary Data Collection and Analysis Software
- 1 each 2.5 mL filling syringe w/ 16 gauge, 8" long needle
- 1 each 100 µL syringe
- 1 each 250 µL syringe
- 1 each burette drive
- Power cord
- USB cable

Optional Accessories

- Computer System
- Printer
- Degassing station

N-ITC III Location

Lift the N-ITC III carefully out of the box and gently place it in the desired work area. As noted earlier, the bench top location of the N-ITC III should accommodate space and power requirements, as well as minimal temperature fluctuations and air currents.

N-ITC III Connections

1. Make sure that the N-ITC III power switch is turned off.
2. Attach the power cord provided to the back of the N-ITC III (Figure 2-1). Do not plug the N-ITC III into a power source at this time.

Figure 2-1: Attaching the N-ITC III power cord and USB cable.

3. Plug the loose USB cable into the back of the N-ITC III (Figure 2-1).
4. Plug the power cord of the N-ITC III into a surge suppressor power strip. Do not turn equipment power on at this time.

Computer Setup

Detailed computer setup instructions are not included here. System configuration may change due to advancements in technology. Follow the instructions included with your system for proper setup and installation.

Connect the free end of the N-ITC III USB cable into a free USB port on the external computer.

Powering the System

Turn the computer power on and allow the system to boot up. Turn on the N-ITC III power. Start the ITCRun software by double-clicking the mouse pointer on the CSC

5300 icon located on the desktop or by going to *Start⇒Programs⇒CSC 5300*. After the software starts set the temperature to 25.0°C and let the system equilibrate for approximately 1 hour to allow the internal electronics to warm up.

Select the "Monitor" tab to display the virtual strip chart. Heat readings (μJ/sec) slowly scroll across from the right side of the screen. The N-ITC III is now ready for use.

Reinstalling Software

The software for your N-ITC III was previously installed at the factory. However, a set of software installation disks are included in the event you need to reinstall the software.

1. Insert N-ITC III software CD in the CD-ROM drive.
2. Select *Start⇒Run*.
3. In the Run dialog box type D:\Setup.exe, or browse to locate the appropriate setup file, and click OK.
4. Follow the on-screen prompts as indicated.
5. When the software installation process is complete, a prompt may appear indicating you need to restart your computer. If this prompt appears then software installation is not complete until you restart your computer system.

Installation of the N-ITC III software is now complete.

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Chapter Three: Theory of Operation

Introduction to Isothermal Titration Calorimetry

There are three ways in which a calorimeter may be designed. Heat measurements may be based on 1) a temperature rise measured in a system of known heat capacity, (ΔT); 2) the measured change in power (typically resistance heating) required to maintain a system at a constant temperature (power compensation); and 3) a direct measure of the heat flowing between the system and large heat sink maintained at a constant temperature (heat flow). Each method (ΔT, power compensation, and heat flow) has its advantages and disadvantages.

The CSC Model 5300 N-ITC III uses a differential power compensation design for maximum sensitivity and responsiveness. Semiconducting thermoelectric devices (TED) are used for temperature control as well as to detect temperature differences between the sample and reference cells. A computer controlled PID loop uses a heater on the sample cell to maintain a zero temperature difference between the sample and reference cells (see Figure 3-1). The power required to maintain this zero difference is used as the calorimeter signal and is monitored as a function of time. If a reaction occurs in the sample cell that produces heat, the heat required to maintain the zero difference decreases by the amount of heat supplied by the reaction, resulting in a peak in the thermogram. A typical experiment consists of several injections spaced minutes apart, resulting in several peaks in the thermogram. The resulting incremental titration curve is

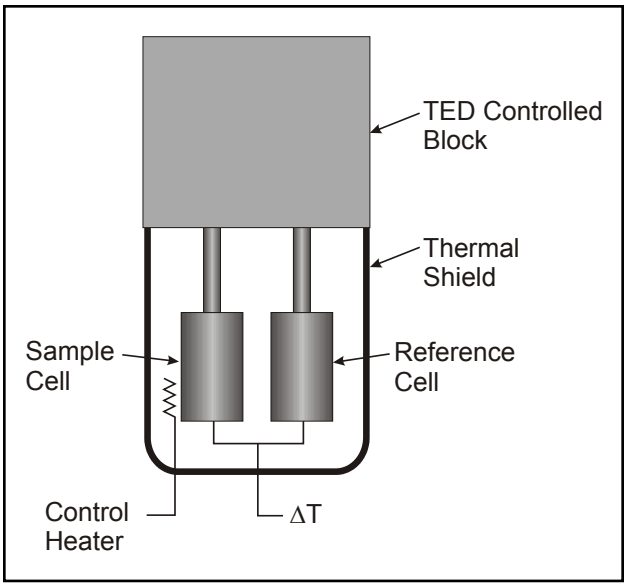

Figure 3-1: Cell Design of the CSC Model 5300 N-ITC III.

used to determine titrate concentration and/or ΔH for stoichiometric reactions. Non-linear regression techniques are used to extract the equilibrium constant (K) as well as ΔH from the thermogram for reactions that do not go to completion.

Applications

Batch/Incremental Titration

In incremental or batch titration, one of the reactants is placed in a syringe or burette external to the reaction vessel. If individual repeated injections are made, incremental titration takes place (as in the example in Figure 3-2); if only one injection is made it is batch injection calorimetry. This generic designation includes direct injection enthalpimetry (DIE).

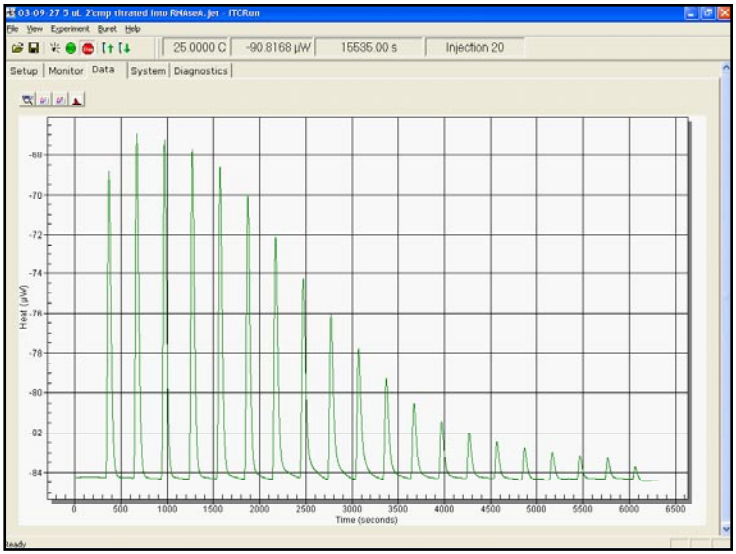

Figure 3-2: Heat flow versus time for an experiment in which a dilute solution of 2'CMP is incrementally titrated into a dilute solution of RNase A.

The baseline data, i.e. heat flow in the regions before and after each titrant pulse, in Figure 3-3 shows the rate of heat loss or gain of the reaction vessel and its contents in the absence of any reaction.

The baseline in this region is a function of heating by stirring, and, in the case of an unsealed vessel, evaporation or condensation. The baseline is used to calculate the area or the heat from each pulse in the reaction vessel during the titration or batch reaction. The thermogram constructed from the integrated peak areas (Figure 3-4) is used for data analysis as described below.

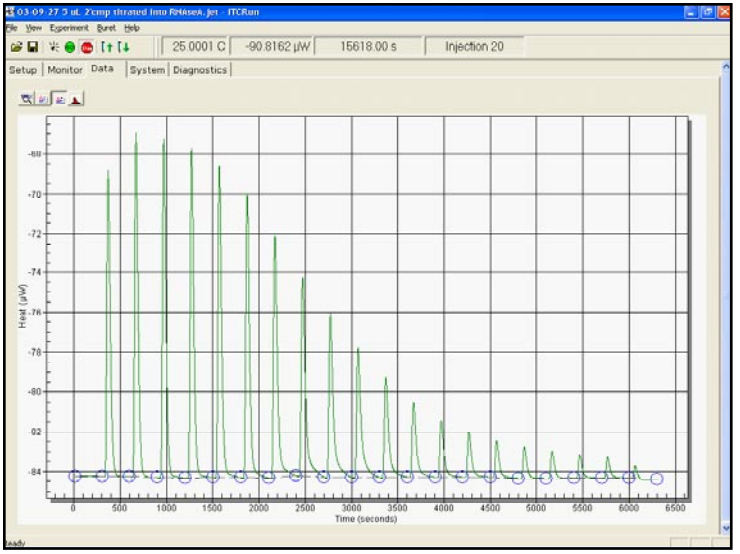

Figure 3-3: Baseline heat outlined along the bottom of the chart which is used when calculating the area of each peak.

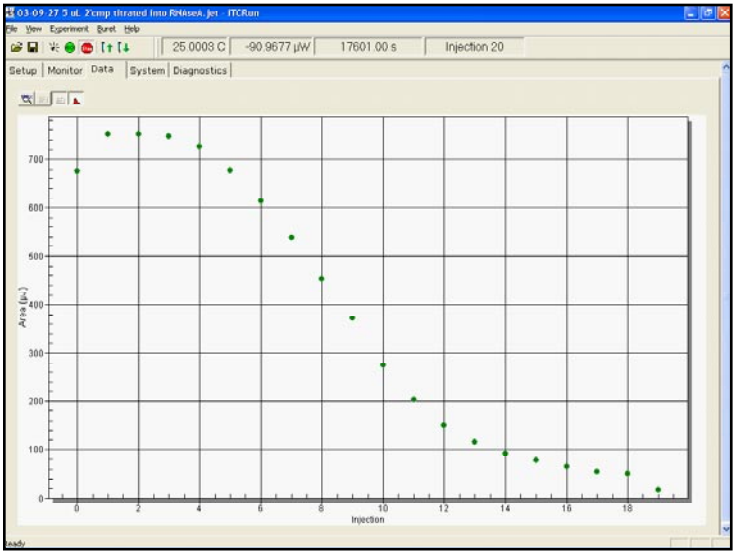

Figure 3-4: Total heat versus time for the incremental titration of a reactant that reacts incompletely with added titrant.

Titration/Data Analysis

A single titration calorimetric experiment yields heat data as a function of the ratio of the concentrations of the reactants. Titration data, in the form of heat change versus volume of titrant added, can be examined for both analytical (thermometric titrimetry) and thermodynamic (titration calorimetry) information.

Other corrections must be made to the heat data to account for heat effects associated with titrant dilution and any temperature difference between titrant and titrate solutions. These corrections are most easily accomplished by performing a blank titration experiment and subtracting the blank heat data from the experimental thermogram.

In the case of quantitative reaction of added titrant, the analysis of the thermogram is quite simple. All peak areas will be the same (with the possible exception of the last peak) and ΔH calculated from the incremental heat and the number of moles of titrant added per increment. The titrant concentration is calculated from the total heat divided by the ΔH for the reaction.

Calculation of Equilibrium Constants

The equilibrium constant for a given reaction may be simultaneously determined with the enthalpy change, if the magnitudes of K and ΔH for the overall reaction taking place in the calorimeter are within certain limits. The family of curves presented in Figure 3-5a

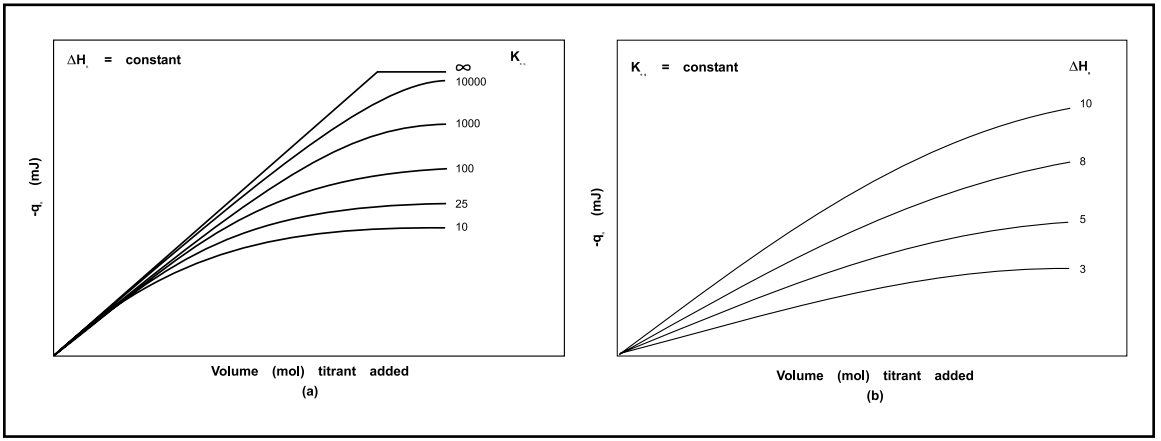

Figure 3-5: Dependence of calorimetric data on (a) K_{eq} and (b) ΔH_R for the titration of A with B.

shows that increased overall curvature of the thermogram is generated with decreasing values of the association constant, K_{eq}.

The total heat observed is directly proportional to ΔH_R, as illustrated in Figure 3-5b. It follows that the lower the K value, the higher ΔH_R must be to calculate K_{eq} and ΔH values with a given reliability.

The limiting values of K and ΔH must simultaneously yield a curved plot of q_R versus moles of titrant and an accurately measured heat.

Mathematical Modeling

Introduction to Equilibrium Models

The thermodynamics of ligands binding to macromolecules have been thoroughly investigated and described. Here we discuss only simple models and present the framework for developing more complex ones. Interested users should refer to the literature for more advanced discussions.

Relating Heat, Enthalpy, and Binding Constants

In a calorimetric experiment, the heat measured for a given titration is equal to the difference in the enthalpy of the system after and before the injection. The system is defined as the contents of the sample cell after the injection, and as the contents of the sample cell plus the material to be injected before the injection. The enthalpy of the system is given as the average excess enthalpy, <ΔH>, times the moles of protein. Thus, the heat observed for the i^{th} injection is given as:

$$q_i = \langle \Delta H \rangle_i C_i V_i \langle \Delta H \rangle_{i-1} C_{i-1} V_{i-1} - \langle \Delta H \rangle_{inj} C_{inj} V_{inj}$$

Equation 3-1

where C is the concentration of protein and V is the volume. If there is no protein in the injection syringe, and assuming ideal solutions (i.e. no dilution heat), then the last term in Equation 3-1 is zero. The cumulative heat at the i^{th} injection is given as:

$$Q_i = \sum_i q_i = \langle \Delta H \rangle_i C_i V_i$$

Equation 3-2

In order to determine binding constants and enthalpy changes from the calorimetric data we need to have a model for the average excess enthalpy terms. The simplest binding model is for one ligand binding to each protein with a binding constant of K and a binding enthalpy of ΔH. K for this system is expressed as:

$$K = \frac{[MX]}{[M][X]}$$

Equation 3-3

where M represents the protein (or any other macromolecule) and X represents the ligand. For this system the protein can exist in two states, either bound or free. By rearranging Equation 3-3 and substituting, the sum of the accessible states of the protein, expressed as concentrations, is given as:

$$[M]_{tot} = [M] + [MX] = [M](1 + K[X])$$

Equation 3-4

Note that $[M]_{tot}$ is the same as C in Equation 3-1. The population of bound protein, P_b, is the proportion of the bound protein to the total protein. Applying Equation 3-3 and Equation 3-4 yields:

$$P_b = \frac{[MX]}{[M] + [MX]} = \frac{K[X]}{1 + K[X]}$$

Equation 3-5

In general, the average excess enthalpy is given as the sum of the population of each state, j, times the change in enthalpy to get to that state, ΔH_j.

For this case then:

$$\langle \Delta H \rangle = \sum_j P_j \Delta H_j = \Delta H \frac{K[X]}{1 + K[X]}$$

Equation 3-6

which can then be substituted into Equation 3-1 or Equation 3-2. Unfortunately, Equation 3-6 is given in terms of the concentration of free ligand, [X], whereas the quantity we control in the experiment is the total concentration of ligand, $[X]_{tot}$. We thus need to express Equation 3-6 in terms of the total ligand concentration.

Again, rearranging Equation 3-3 and substituting, the total concentration of ligand is given as:

$$[X]_{tot} = [X] + [MX] = [X](1 + K[M])$$

Equation 3-7

Combining Equation 3-7 with Equation 3-4 gives a quadratic equation which can be solved to express [X] in terms of the total concentrations of ligand and macromolecule:

$$[X] = \frac{-1 - K([M]_{tot} - [X]_{tot}) + \sqrt{(1 + K([M]_{tot} - [X]_{tot}))^2 + 4K[X]_{tot}}}{2K}$$

Equation 3-8

This can then be substituted into Equation 3-6 to give an analytical expression for q_i or Q_i for each injection. If we have multiple sites which are identical and independent we simple multiply $[M]_{tot}$ (Equation 3-4) by N, the number of sites.

Notice that the value of N is equal to the exponent of the concentration of the free ligand in Equation 3-3. In a system where the binding ratio is one to one (i.e., N=1) the exponent is implied, but expressing the binding reaction in general terms for any value of N gives:

$$M + NX = MX_N$$

Equation 3-9

Hence, the equilibrium constant for the overall reaction is:

$$K_{eq} = \frac{[MX_N]}{[M][X]^N}$$

Equation 3-10

When N=1, the binding constant, K, is equal to the equilibrium constant, K_{eq}. Owing to this relationship it should be noted that even if the known stoichiometry is one binding

site per protein based on structural information, the experimental value of N may still vary due to error in protein concentration estimation or to degradation of the protein sample. For experiments performed under conditions in which K can be determined, the value of N does not affect the fitted value of K or ΔH.

Consider the case in which we have two independent binding sites on the protein, but each site has a different binding constant. The sum of the accessible states of the protein, expressed as concentrations, is then given as:

$$\begin{aligned}[M]_{tot} &= [M] + [M_1X] + [M_2X] + [MX_2] \\ &= [M]\left(1 + K_1[X] + K_2[X] + K_1K_2[X]^2\right) \\ &= [M](1 + K_1[X])(1 + K_2[X])\end{aligned}$$

Equation 3-11

The average excess enthalpy is then:

$$\begin{aligned}\langle \Delta H \rangle &= \sum_j P_j \Delta H_j \\ &= \frac{\Delta H_1 K_1[X]}{(1 + K_1[X])(1 + K_2[X])} + \frac{\Delta H_2 K_2[X]}{(1 + K_1[X])(1 + K_2[X])} + \frac{(\Delta H_1 + \Delta H_2) K_1 K_2[X]}{(1 + K_1[X])(1 + K_2[X])}\end{aligned}$$

Equation 3-12

which can be substituted into Equation 3-1 or Equation 3-2. In this case we need to solve a cubic expression for [X]. This can be readily accomplished using standard numerical algorithms and the result substituted into Equation 3-12.

More complex models are developed by the same approach. Based on the model an expression is derived for the average excess enthalpy in terms of the free ligand concentration, and then the free ligand concentration is expressed in terms of the total concentrations of ligand and protein generally requiring numerical methods to obtain a solution.

The following references give further detail on modeling of titration data or general binding phenomena:

Freire, E., Mayorga, O. L. & Straume, M. (1990). Isothermal Titration Calorimetry. *Anal. Chem.* **62**, 950A-959A.

Wiseman, T., Williston, S., Brandts, J. F. & Lin, L.-N. (1989). Rapid Measurement of Binding Constants and Heats of Binding Using a New Titration Calorimeter. *Anal. Biochem.* **179**, 131-137.

Wyman, J. & Gill, S. J. (1990). *Binding and Linkage: The Functional Chemistry of Biological Macromolecules* (University Science Books, Mill Valley).

Fitting of Calorimetric Data

Values for K and ΔH are obtained by fitting the integrated heats from a titration experiment to Equation 3-1 or Equation 3-2. Use of Equation 3-2 is simpler and faster as it requires only one calculation of the excess enthalpy for each injection whereas Equation 3-1 requires two. In general, use of Equation 3-2 presents no problem, however by using Equation 3-2, the error from all the previous injections is propagated into each value of the cumulative heat. Better statistics are therefore obtained by fitting the individual heats according to Equation 3-1.

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Chapter Four: Hardware Description

Description of Parts and Functions

The CSC Model 5300 N-ITC III consists of the measuring unit (calorimeter block and two non-removable reaction vessels), the burette assembly, which includes the stirring system, and a cleaning accessory. With the exception of the power on/off switch located on the back of the calorimeter unit, all functions of the N-ITC III are controlled remotely by the computer through the USB connection. For more information see Chapter Five: Software Description.

Measuring Unit

The measuring unit includes the calorimeter block and two non-removable reaction vessels (sample and reference cells). Access tubes extend downward from inside the burette mounting cavity on the top of the calorimeter and serve as conduits for the filling syringe, titrant delivery, and reference needle. They also provide for titrant equilibration and as a thermal barrier to the environment outside the calorimeter.

The CSC Model 5300 N-ITC III uses a differential power compensation design for maximum sensitivity and responsiveness. Semiconducting thermoelectric devices (TED) are used for temperature control as well as to detect temperature differences between the sample and reference cells. A computer controlled PID loop uses a control heater on the sample cell to maintain a zero temperature difference between the sample and reference cells (see

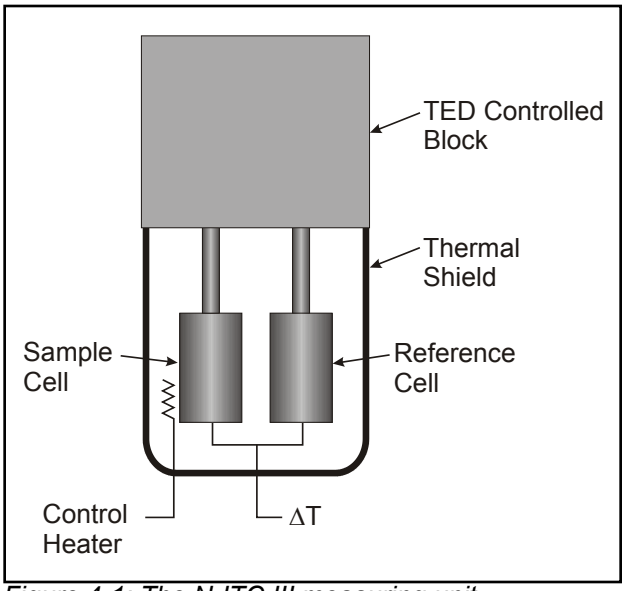

Figure 4-1: The N-ITC III measuring unit.

Figure 4-1). The power required to maintain this zero difference is used as the calorimeter signal and is monitored as a function of time. If a reaction occurs in the sample cell that produces heat, the heat required to maintain the zero difference decreases by the amount of heat supplied by the reaction, resulting in a peak in the thermogram.

A calibration heater located on the outside of the sample cell is used to provide precisely controlled heat pulses for electrical calibrations and to verify instrument performance.

The entire measuring unit is encased within an insulated air-tight canister which has been purged on a vacuum pump and filled with dry nitrogen at the factory. This is to prevent possible condensation and evaporation of moisture around the unit which would create excessive baseline noise.

The purge port valve on the back of the N-ITC III should remain in the closed position at all times to maintain the integrity of the nitrogen purge.

Reaction Vessel

The calorimeter uses two 1.0 mL reaction vessels. The reaction vessels are made of 24K gold with platinum access tubes. The reference cell is constructed to match as closely as possible the thermal properties of the sample cell. Accordingly, a reference needle is placed inside the reference cell during operation to correspond to the titrant needle in the sample cell.

Syringe/Stirrer

Two burette syringes are provided at 100 µL and 250 µL capacities, respectively. The only difference in dimensions between the two is the inner diameter of the syringe barrel, since the outer dimensions must be identical for proper installation in the burette.

The titrant syringe needle also functions as the stirrer and extends down into the reaction vessel from the top when the burette is mounted. The needle is balanced for optimum stirring efficiency, and has two Teflon bushings to help dampen stirring noise and ensure that the needle spins true within the cell access tube (Figure 4-2).

Extreme care should be taken not to bend the syringe needle because this would impair proper stirring and possibly damage the reaction vessel.

Each syringe needle is equipped with a flattened, slightly twisted paddle at the tip which does the actual stirring of the solutions in the cell. The stirring paddle spins clear of the sides of the reaction vessel. When stirring is activated the contents of the reaction vessel are stirred continuously until the end of the experiment or until stirring is turned off.

Stirring is controlled by a stepping motor mounted inside the calorimeter. This type of motor is used because of its very constant and adjustable speed. The motor drives the rotating shaft of the burette which holds the titrant syringe, and torque is transmitted to the stirring paddle through the syringe needle.

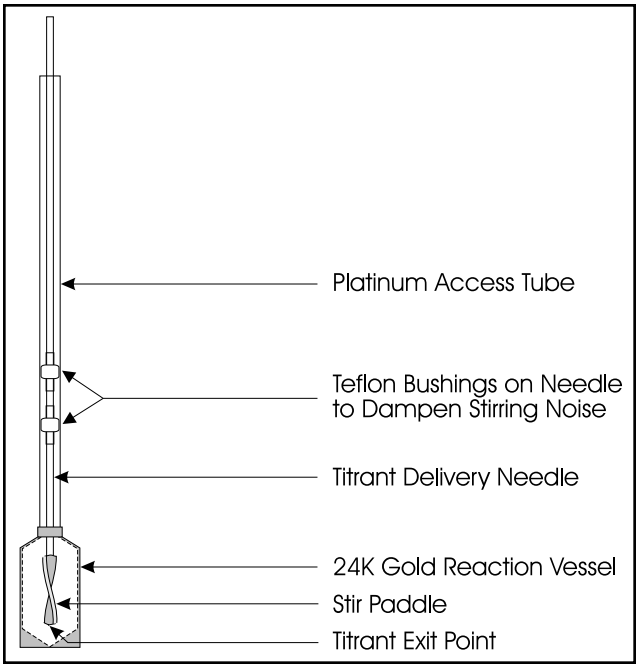

Figure 4-2: The sample cell assembly.

Burette Assembly

The burette functions to accurately deliver the titrant to the reaction vessel at user specified volumes and intervals. The assembly doubly functions as the stirring mechanism for the reactants in the cell when the titrant syringe is installed. The rotating shaft on the lower portion of the burette assembly holds the titration syringe in place, and has two external O-rings which provide the friction necessary for the stir motor to rotate the shaft during operation.

When the syringe is filled with titrant, the plunger and barrel are carefully inserted into the

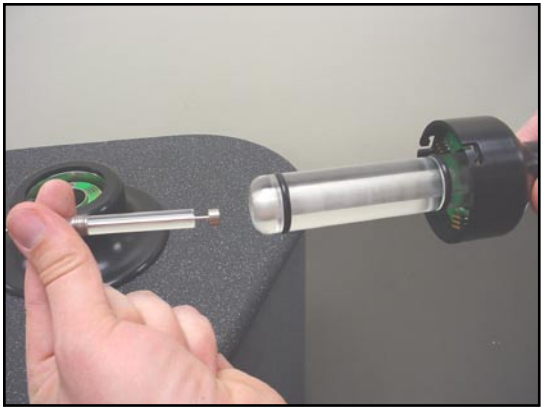

Figure 4-3: Inserting the syringe into the burette shaft.

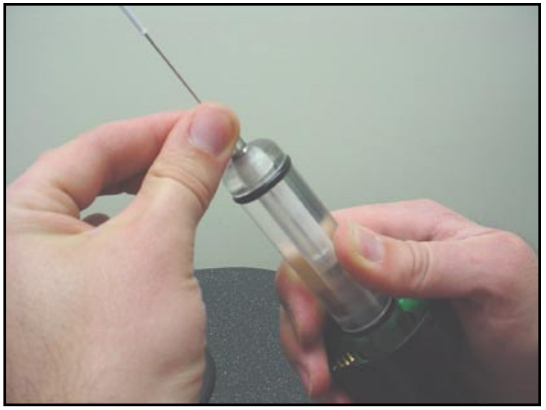

Figure 4-4: Finger-tightening the syringe in the burette shaft.

rotating shaft of the burette assembly (Figure 4-3). The rotating shaft on the burette is held secure in one hand, and using the knurled knob at the base of the syringe barrel, the syringe is finger-tightened into place with the other hand (Figure 4-4).

The top portion of the burette handle displays a graduated scale with an indicator showing the relative position of the syringe plunger during an experiment. Note that the indicator must be in the fully raised position before installing a loaded syringe into the burette. For more information on lowering and raising the plunger, refer to Chapter 5: Software Description.

The bell-shaped upper portion of the burette assembly has three notched keys for correct orientation in the instrument. The burette is installed by carefully guiding the shaft, needle first, into the top opening of the calorimeter (Figure 4-5) and lining these notches up with the three locking posts located in the mounting ring at the top. The burette handle is then gently pressed downward, rotated slightly clockwise, and released to secure the burette in place. Removal of the burette from

Figure 4-5: Inserting the burette assembly into the calorimeter.

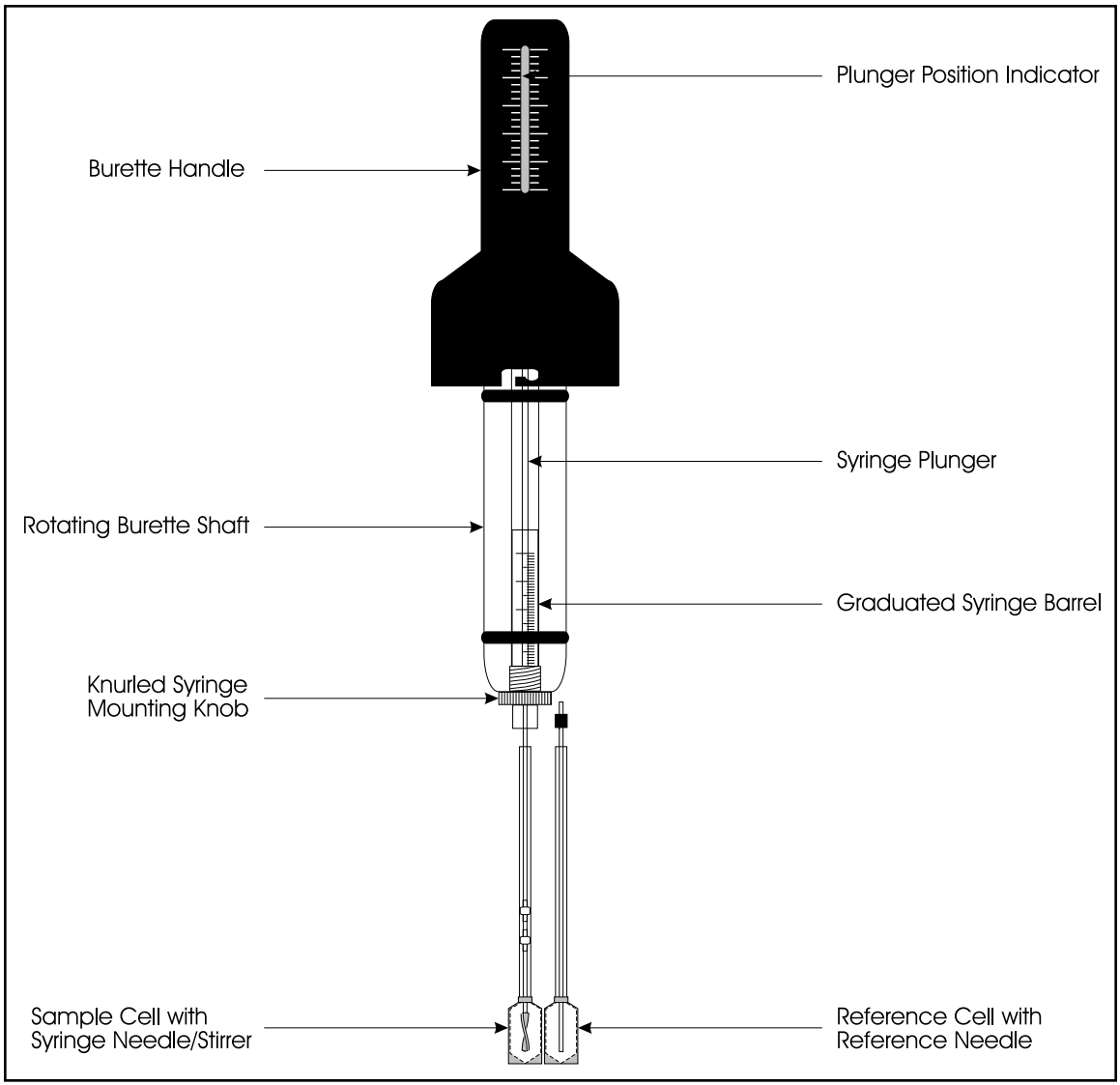

Figure 4-6: Relative positions and orientation of the burette, syringe, needles, and cells during an experiment.

the calorimeter is the reverse of this process. When the burette is installed properly, the circular contact boards at the burette/calorimeter interface provide electrical power to the burette and enable the functional control necessary to perform a titration.

Figure 4-6 shows the relative positions of the burette, syringe, needles, and cells during an experiment.

USB Connection

A Universal Serial Bus connector located on the rear of the instrument (Figure 4-7) allows remote operation of the calorimeter, and is USB 1.1 compliant.

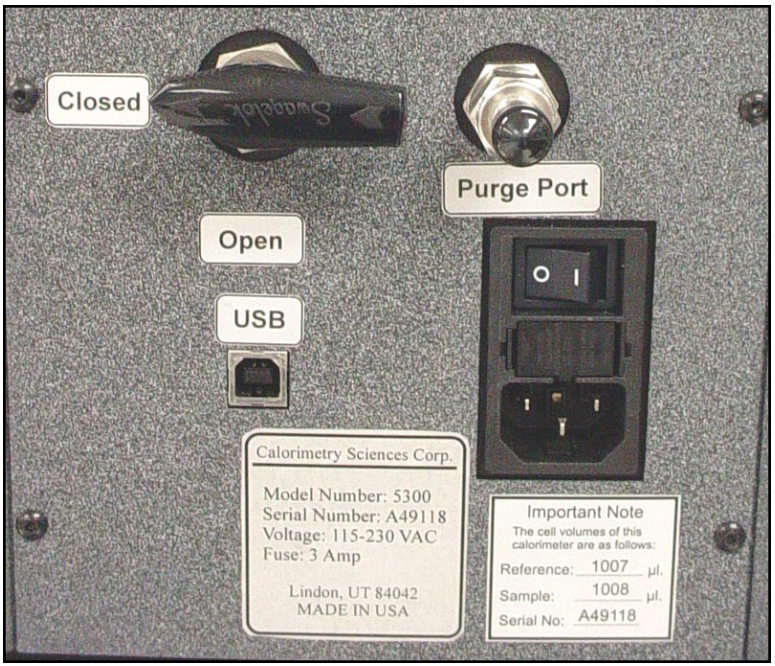

Figure 4-7: Rear panel of the N-ITC III showing the location of the USB port, power switch and power cord socket, gas purge port, and purge port valve.

Power Supply

The calorimeter has an autosetting power supply which may be used with input voltage ranges of both 90-130 VAC and 180-260 VAC, 47-63 Hz. No manual changes to the instrument are required when switching from one power source to another, except for the frequency setting in the ITCRun software (see Chapter 5: Software Description). The power supply has four DC outputs which provide voltages for the calorimeter electronics, and has an internal fuse to protect against shorts or excessive loads.

Cleaning Tool

A scrupulously clean sample cell is essential in order to obtain meaningful titration data. Because the reaction vessel is non-removable, the filling syringe may be used to repeatedly flush the cell several times; or, when more rigorous cleaning is called for, a cleaning adapter is provided which allows the user to easily flush large volumes of fluid through the cell (Figure 4-8). The cell should be cleaned immediately following an experiment, then rinsed with buffer to condition it for the next experiment. The cells should be filled with pure deionized water between experiments to prevent contamination from drying to the cell walls.

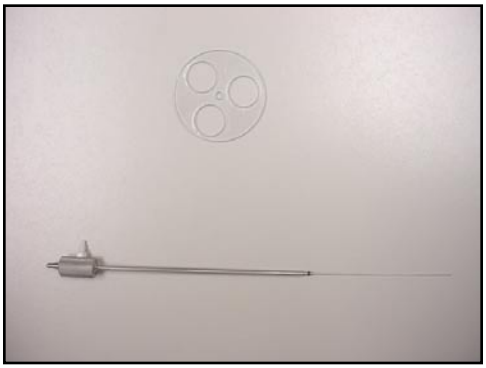

Figure 4-8: The 5300 cleaning adapter and support disk.

The cleaning tool is used as follows:

1. Remove the burette assembly and syringe from the top opening of the N-ITC III, and withdraw the cell contents using the filling syringe.
2. Place the cleaning tool support disk in the circular top of the calorimeter (Figure 4-9). The support disk helps to guide the cleaning

Figure 4-9: The support disk.

adapter into the sample cell, and prevents side-to-side movement which could bend the tool and damage the cell.

Do not use the cleaning adapter without the support disk in place as this may cause the adapter needle to bend and possibly damage the reaction vessel.

3. Moisten the O-ring on the shaft of the cleaning tool just enough to lubricate. Insert the tool assembly into the center hole of the support disk, and carefully lower the shaft into the cell opening (Figure 4-10). When the O-ring enters the cell opening, continue lowering the tool with a slight rotation to work the O-ring down into the neck of the sample cell access tube.

Figure 4-10: Inserting the cleaning tool.

4. Connect a length of 1/16 inch I.D. Manosil silicone rubber tubing provided to the side port of the cleaning tool, and place the other end of the tube in a beaker of clean deionized or distilled water. Connect another length of tubing to the top port leading to a vacuum flask which is connected to a vacuum pump (Figures 4-11 and 4-12).

Figure 4-11: Connecting the tuning to the cleaning tool.

Figure 4-12: The assembled cleaning apparatus.

5. Apply a vacuum to draw the water through the system and flush the cell. Figure 4-13 describes the flow of water through the apparatus.

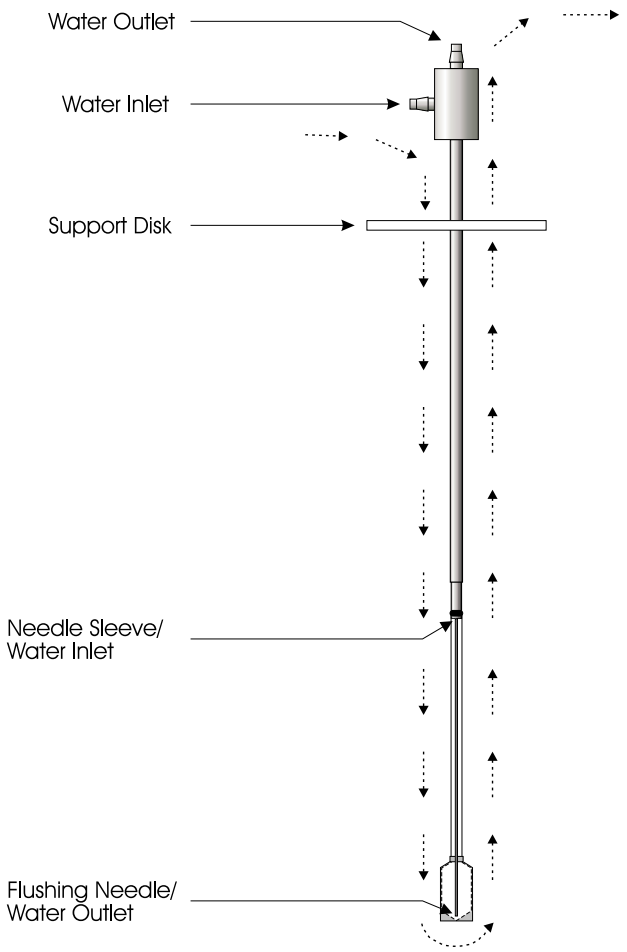

Figure 4-13: Sideview of the cleaning tool in the sample cell. Water is drawn into the side port inlet and down the length of the inner needle where it exits near the bottom of the cell. The water then flows upward around the needle the length of the cell and access tube where it enters the outer sleeve of the cleaning tool just below the O-ring. Flow continues upward to the outlet at the top of the tool and out to the vacuum flask.

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Chapter Five: Software Description

Overview

Software for the N-ITC III consists of two separate sections. A stand-alone acquisition program called ITCRun controls experiment setup and data collection, while BindWorks provides a full-featured data analysis program.

ITCRun Software Installation

The software for your Isothermal Titration Calorimeter was previously installed at the factory. However, a software installation CD is included in the event you need to reinstall the software.

To reinstall the software:

1. Insert ITCRun CD in the CD-ROM drive.
2. From Windows, select *Start⇒Run*.
3. In the Run dialog box type: D:\Setup.exe (or browse to locate the appropriate drive and folder containing the ITCRun setup file) and click OK.
4. Follow the on-screen prompts as indicated.
5. When the software installation process is complete, a prompt may appear indicating you need to restart your computer. Software installation is not complete until you restart your computer system.

Installation of the ITCRun software is now complete.

General

To load the data acquisition software, from the Windows desktop select *Start⇒Programs⇒ITCRun*, or double-click the ITCRun icon located on the desktop (Figure 5-1).

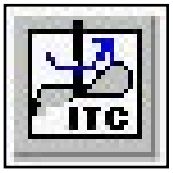

Figure 5-1: ITCRun icon.

ITCRun will initialize and the data acquisition screen shown below will appear (Figure 5-2). The key features of this display are also shown for reference. Detailed descriptions of the operations associated with these features follow.

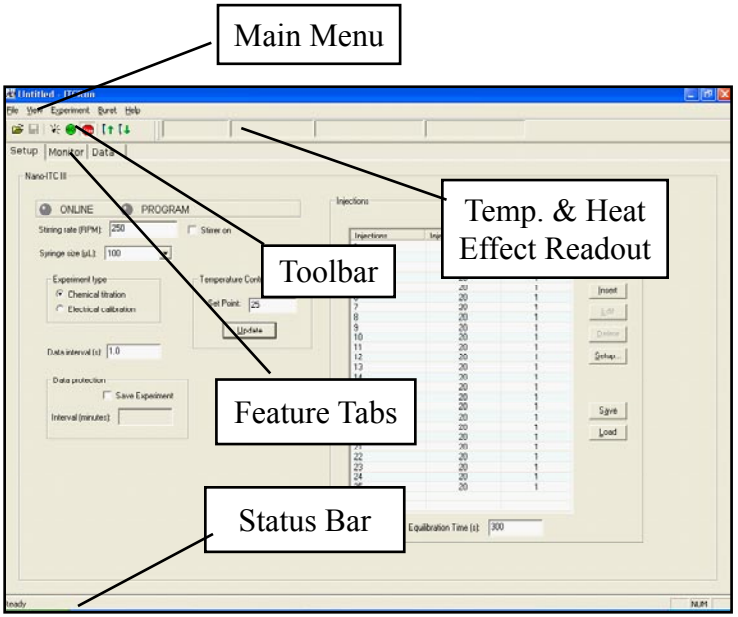

Figure 5-2: The initial ITCRun program display.

Main Menu

The main menu contains basic commands used for setup and control of the instrument and its associated displays.

File Menu

Figure 5-3 shows the file menu. The file menu commands are described below.

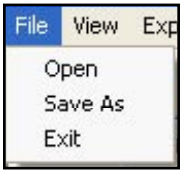

Figure 5-3: The File Menu.

File • Open

Opens an existing set of scan data. Use this command to open an existing

data file for review. Also appears on the toolbar as . Shortcut Key: CTRL+O

File • Save As

Saves the current data file to a specified file name. Use this command to save and name (or rename) the current data file. To save a document using its existing name and directory, use the icon on the toolbar.

File • Exit

Exits the ITCRun program. Use this command to end your ITCRun session. ITCRun prompts you to save documents with unsaved changes. If the N-ITC III is currently performing an experiment (non-idle mode), it will ask you to confirm your intention to exit the program. If you still wish to exit while ITCRun is collecting data, it will prompt you to save the data file (if any). Shortcut Keys: ALT+F4

View Menu

Figure 5-4 shows the view menu. The view menu commands are described below.

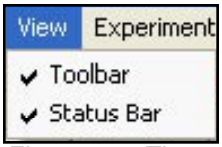

Figure 5-4: The View Menu.

View • Toolbar

The View Toolbar command controls the appearance of the function icons toolbar on the display. When this option is selected, the toolbar is present. When the option is deselected, the toolbar is removed from the screen.

View • Status Bar

The View Status command toggles the status bar on the bottom of the screen in the same manner as the View Toolbar command.

Experiment Menu

Figure 5-5 shows the experiment menu. The experiment menu

Figure 5-5: The Experiment Menu.

commands are described below.

Experiment • Settings

The Experiment Settings command opens the settings dialog box (Figure 5-6). Also appears on the toolbar as .

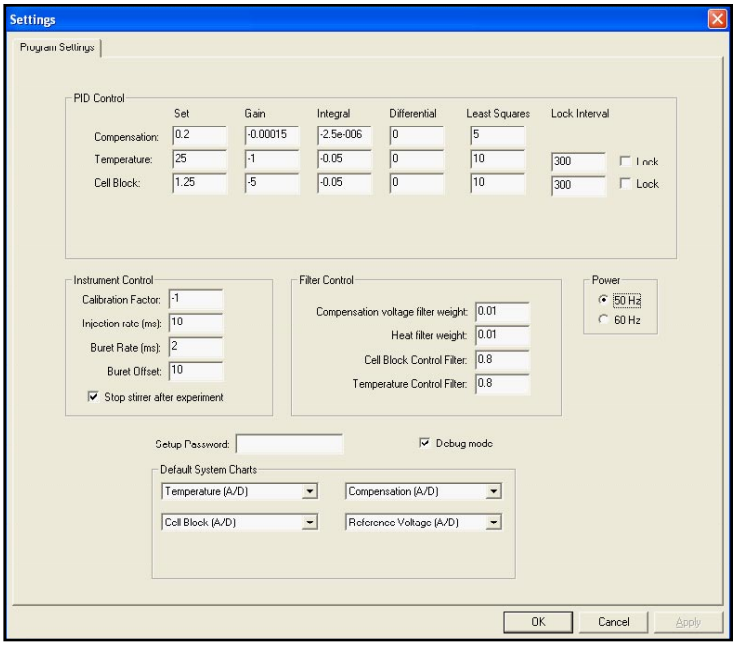

Figure 5-6: The Settings Dialog Box.

The *PID Control* portion of the settings screen controls the N-ITC III temperature set points and PID loop variables. The user should not change any of the parameters in this area with the exception of the Temperature Set variable, which shows the current operating temperature of the N-ITC III.

The *Instrument Control* section contains settings for the calibration and buret control. The Injection rate (ms) setting controls the speed of the buret stepper motor during the injection in terms of milliseconds per step. The number of steps corresponds to the injection volume and syringe size according to the relationship:

$$\text{Steps} = \frac{\text{Injection Volume}}{\text{Syringe Size}} \text{ x } 7000$$

Equation 5-1

The duration of the injection is the number of steps multiplied by the injection rate setting. For example, if a 100 µL syringe is used to make an injection of 5 µL, and the injection rate setting is 35, the duration of the injection is:

$$\left(\frac{5\,\mu L}{100\,\mu L}\ \text{x}\ 7000\right)\ \text{x}\ 35\ \text{ms/Step} = 12250\ \text{ms} = 12.25\ \text{seconds}$$

Equation 5-2

The actual injection rate in terms of volume, therefore, is 5 µL/12.25 s = 0.408 µL/s.

The settings in the *Filter Control* area of the Settings window should not be changed by the user. The *Power* setting should indicate the proper frequency of the local line voltage. The user may define a *Setup Password* to limit access to the Settings dialog box. When the *Debug* option is selected, two more feature tabs appear on the main screen display: the System feature tab and the Diagnostics feature tab (see Feature Tabs below). The display of the System tab is controlled by the *Default System Charts* portion of the Settings window when *Debug* is selected.

Experiment • Start

The Experiment Start command begins data collection for an experiment. When an experiment begins, the user will be prompted to enter a file name to which the data will be saved in real time. Also appears on the toolbar as .

Experiment • Stop

The Experiment Stop command is used to abort data collection before the completion of the experiment protocol. Also appears on the toolbar as .

Buret Menu

Figure 5-7 shows the buret menu. The buret menu commands are described below.

Buret • Move Up

The Buret Move Up command moves the buret to the fully raised position. Also appears on the toolbar as .

Figure 5-7: The Buret Menu.

Buret • Move Down

The Buret Move Down command moves the buret to the fully lowered position. Also appears on the toolbar as .

Help Menu

Figure 5-8 shows the help menu. If a help file is not available, it will be implemented in later revisions of ITCRun.

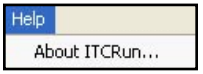

Figure 5-8: The Help Menu.

Help • About ITCRun...

The Help About ITCRun command displays the software version number and other information about ITCRun.

Feature Tabs

Feature tabs control all operations of the Isothermal Titration Calorimeter. These tabs are used to set up key parameters of your experiment and aid in the automation of the experiment process.

Setup Tab

Figure 5-9 shows the Setup tab. This tab establishes general experiment parameters. Change or modify the values to suit your requirements. Experiments should be set up based on the expected results. Typically, experiments will be twenty injections of 5 µL when using the 100 µL syringe, and twenty-five injections of 10 µL when using the 250 µL syringe. To collect data in either titration mode or calibration mode, single-click the Go icon from the toolbar, or select *Experiment⇒Start* from the Main Menu. The user will be prompted for an experiment filename, and the program indicator on the Setup tab will turn red indicating that the program has begun.

Stirring Rate (rpm) & Stirrer On

This number should typically remain at 150 rpm, although rates of 200-250 rpm may also

be used. Click on the stirrer on box to turn on stirring.

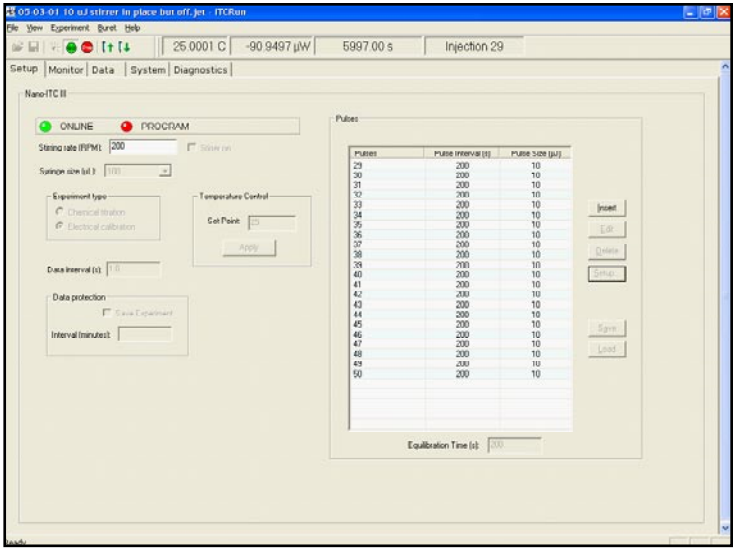

Figure 5-9: The Setup Feature Tab.

Syringe Size (µL)

Syringe size (either 100 µL or 250 µL) for the current titration.

Experiment Type

Choose chemical titration or electrical calibration. If chemical titration is chosen, choose the injection volume in µL needed for the experiment. If electrical calibration is chosen, choose the pulse size in µJ.

Temperature Control – Set Point (°C)

This sets the control temperature of the ITC. After changing the set point you must click Apply to incorporate the change.

Data Interval (s)

Time between data points collected. The default value is 1 second.

Equilibration Time (s)

Specifies a time, in seconds, to collect data before the first injection or electrical pulse occurs. This option is typically used to establish a baseline.

Setup Button

Used to enter the basic parameters of an experiment (Figures 5-10 and 5-11). The parameters are as follows:

- *Injection or Pulse Interval*

  The time interval, in seconds, between injections or electrical pulses.

- *Injection Volume or Pulse Size*

  The size in µL for an injection or µJ for a pulse.

- *Number of Injections or Pulses*

  The number of injections or electrical pulses required for the experiment.

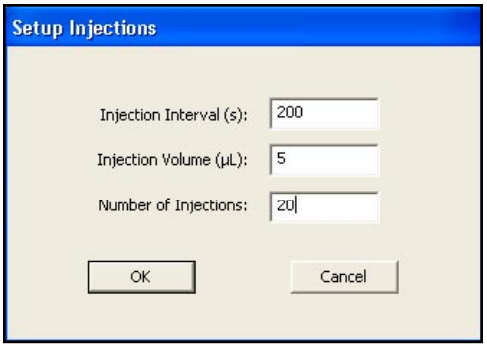

Figure 5-10: Injection Setup.

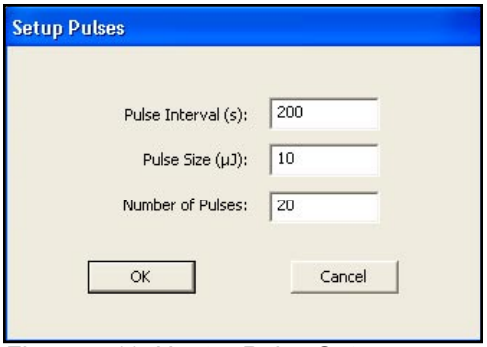

Figure 5-11: Heater Pulse Setup.

Monitor Tab

Figure 5-12 shows the Monitor tab. This tab allows the user to view data being collected by the N-ITC III on a virtual strip chart. When you first start the ITCRun program, data from the ITC scrolls slowly across the screen **but is not saved**. Only when the *Experiment⇒Start* button is clicked or the Go icon is chosen, will the displayed data be saved to a file. The Monitor strip chart is used so that the user can watch this unsaved data in order to judge when the N-ITC III is equilibrated or ready to begin an experiment.

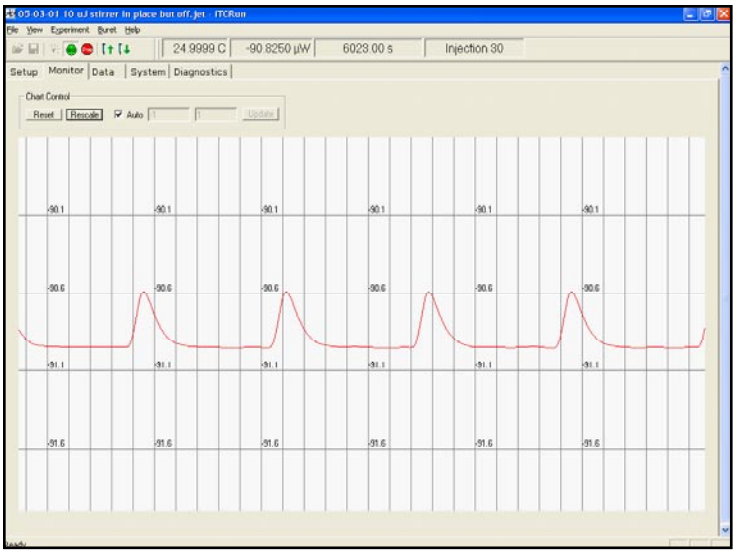

Figure 5-12: The Monitor Feature Tab.

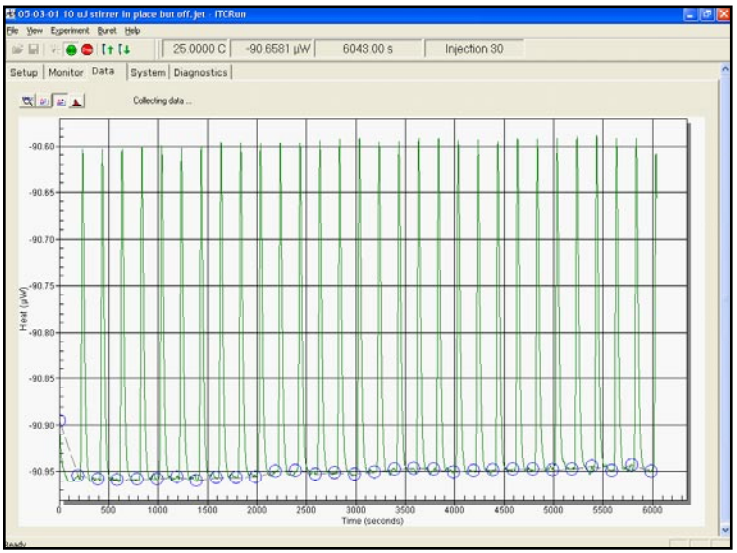

Figure 5-13: The Data Feature Tab.

Reset chart

This option completely clears the chart of data. Choosing this option does not abort or stop the experiment.

Rescale chart

This option will autoscale the y-axis on the virtual strip chart.

Auto

This option allows the user to manually scale the chart to the values shown in the two text boxes to the right of the Auto check box.

Data Tab

Figure 5-13 shows the Data tab. This tab displays the current data in any of several formats for convenience during data collection. The graph will be updated during the experiment for each data point generated.

Zoom

Any portion of the data shown

on the data tab my be enlarged by holding down the left mouse button and dragging a window around the desired region. To restore the full data display click once on the "Zoom Out" icon which appears above the display as .

Show baseline

Appears on the display as . This icon displays a basic baseline that follows the contours of the data.

Subtract baseline

Appears on the screen as . Selecting this icon visually corrects the displayed data for baseline drift. There is no actual change in the data file itself. Deselecting the icon will restore the actual data display.

Show Area

Appears on the toolbar as . This icon displays the integrated areas of each peak in μJ.

System Tab and Diagnostics Tab

The System and Diagnostics feature tabs are used for evaluation purposes by CSC technicians and only appear when the "Debug" option is selected in Settings.

Performing a Titration using ITCRun

Refer to Chapter 4: Hardware Description for additional information about preparing the sample and reference cells, mounting the burette drive and syringe, and establishing a stable baseline with the stirrer rotating.

1. Using the filling syringe, flush the sample cell several times using the same buffer solution in which the sample is prepared. After flushing, remove all of the buffer and load the sample into the sample cell (middle access tube) inside the N-ITC III (Figure 5-14). Complete loading will take approximately 1.3 mL of solution. Fill the cell

Figure 5-14: The filling syringe used to flush and fill the cells.

slowly to allow air bubbles to evacuate through the top of the cell. When liquid is just visible at the opening of the access tube, continue to apply positive pressure to the syringe while slowy withdrawing it from the cell to maintain the fill level and prevent new bubbles from being introduced into the cell (Figure 5-15a). When using aqueous solutions the reference cell (side access tube) should be filled with water. Make sure that the reference needle is inserted into the reference cell access tube after filling (Figure 5-15b).

2. Load the 100 µL syringe with the ligand solution making sure to remove any bubbles from the barrel of the syringe, but leaving a small 5 to 10 µL air gap between the plunger tip and the liquid in the barrel. Wipe the needle with a tissue and

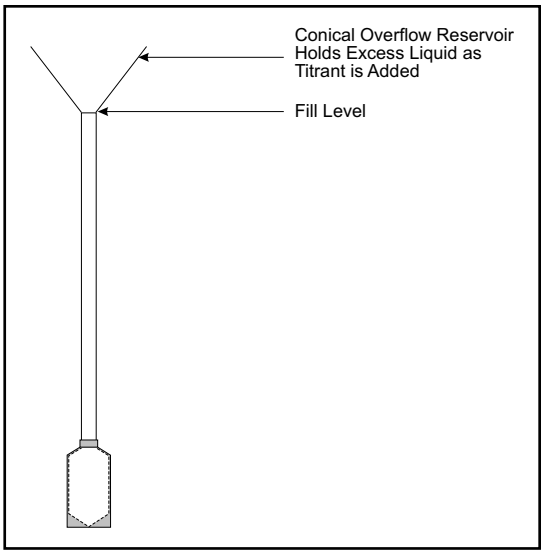

Figure 5-15a: Side view of sample cell and access tube showing fill level and overflow reservoir.

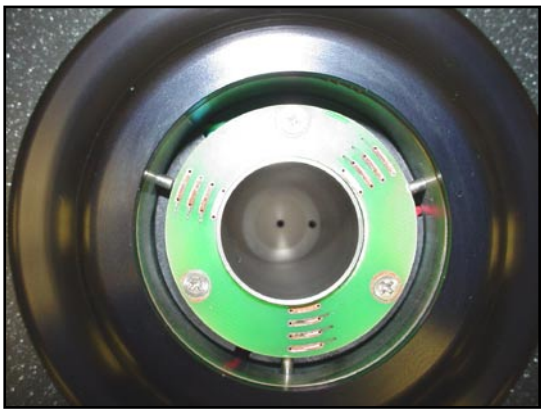

Figure 5-15b: Sample (center) and reference (right side) cell access tubes. Liquid should be just visible at the bottom of the conical overflow reservoir when the cell is filled.

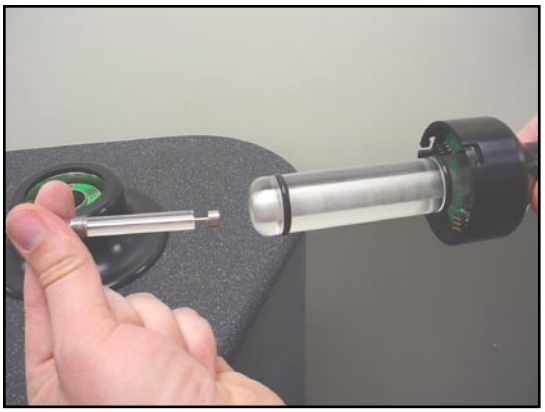

Figure 5-16: Inserting the syringe into the burette shaft.

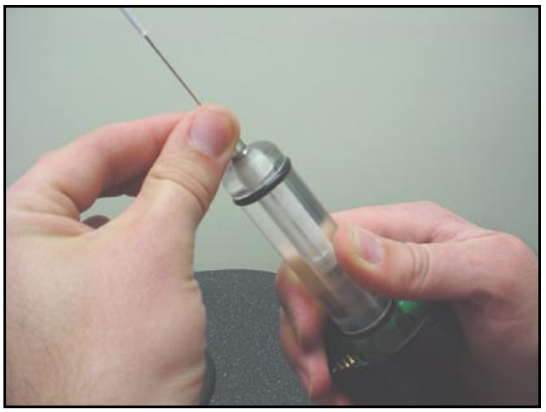

Figure 5-17: Finger-tightening the syringe in the burette shaft.

then screw the syringe completely into the buret drive as shown in Figures 5-16 and 5-17.

3. Insert the syringe and burette drive into the N-ITC III as shown in Figure 5-18. Make sure that the burette key slots line up correctly with the syringe position slot located towards the front of the N-ITC III. Push the drive completely down and turn clockwise to lock in place.

4. Turn on the stirrer at 150 rpm and allow the system to re-equilibrate until the heat reading on the calorimeter is stable.

5. Set the parameters for the particular experiment of interest in ITCRun under the Setup tab (Figure 5-19).

6. Click the Go icon or select *Experiment⇒Start* to begin the titration. While the program is running the

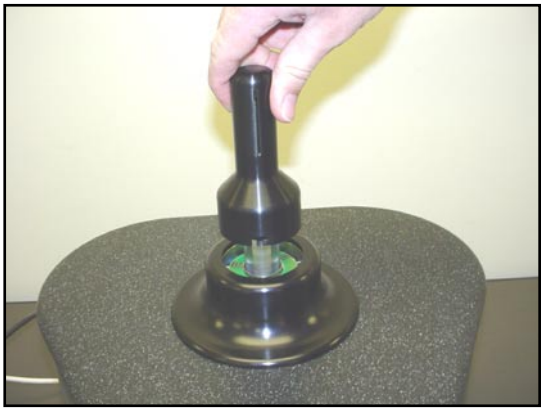

Figure 5-18: Inserting the burette assembly into the calorimeter.

Program indicator in ITCRun will flash.

7. When the titration has concluded, select *File⇒Save* to store the data to disk.

8. Evaluate the data using the Bindworks software package.

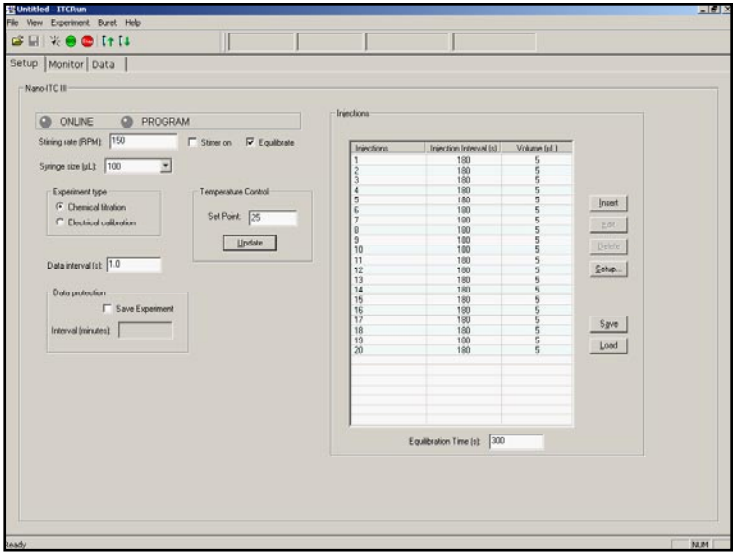

Figure 5-19: The Setup tab showing experiment parameters.

BindWorks

Overview

BindWorks is a computer program that aids in analysis and revision of data derived from titration calorimeters.

BindWorks offers the following features:

- Quick, easy integration of heat rate data from Isothermal Titration Calorimeters.
- Graphing of both Cumulative and Peak heats.
- Correction for heat of dilution and blank effects.
- Editing of individual data points.
- Data import and export to other software programs.
- “Copy and Paste” graphs to other Windows-based programs.
- Creation of BindWorks compatible data files.

Getting Started

Installing BindWorks

Before installing BindWorks close any other "Visual" applications. These include programs written in Visual Basic, Visual C, or other similar applications. If visual applications are running on your system when you try to install BindWorks an error message will result. It is only necessary to close these applications during the install process. You may re-open these applications after completing the BindWorks installation procedure.

To install BindWorks, start Microsoft Windows, then insert the BindWorks distribution CD in the disk drive. Select *Start⇒Run*. In the Command Line box, type D:\SETUP and press ENTER, or browse to locate the appropriate folder and setup file.

BindWorks prompts you for a path name for the program. We suggest you use the default specification if possible. If you desire a path name other than the default, enter the new path name in the setup box and press ENTER.

Using BindWorks

Select *Start⇒Programs⇒BindWorks⇒BindWorks* to run BindWorks. Analysis of data acquired by the CSC N-ITC III requires three steps:

1. Manipulation of raw data.
2. Integration of the peaks.
3. Modeling and fit of data.

Manipulation of raw data

Once the calorimeter has acquired experiment data it may be necessary to manipulate the data or baseline slightly to minimize any anomalies. Several easy-to-use features make manipulation of data quick and simple. To load a raw data file into BindWorks:

1. Select the Raw Data tab.

2. Select *File⇒Open* from the main menu.
3. Locate the data file from the appropriate file folder and click OK.
4. The data file is loaded into BindWorks. A titration data file of RNase A – 2’ CMP is shown in the example.

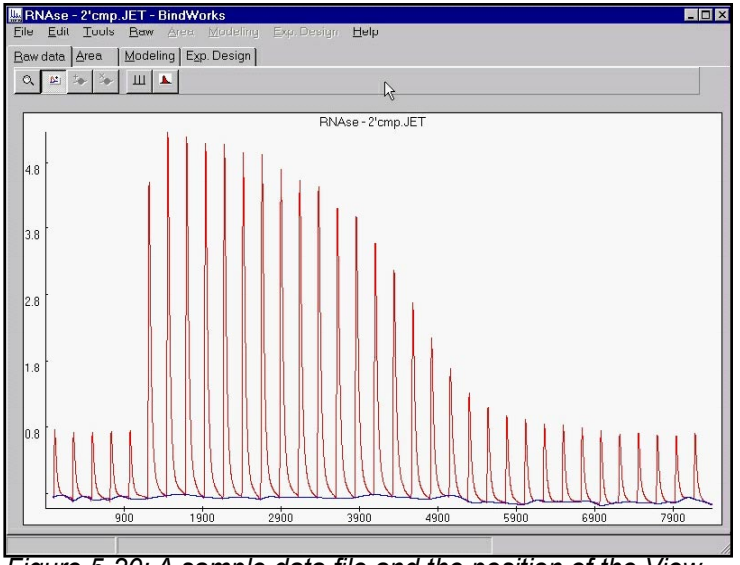

Figure 5-20: A sample data file and the position of the View Baseline icon.

When your data file is loaded, BindWorks will create a baseline based on the data before and after the injection. To view the calculated baseline single-click the View Baseline icon from the toolbar. The calculated baseline will appear, superimposed in blue on your data set (Figure 5-20).

If the baseline is unsuitable, single-click the Zoom icon on the toolbar. BindWorks will parse your data, label each injection, and add nodes to the calculated baseline (Figure 5-21).

A simple, intuitive series of data “markers” denote the current location of a sliding zoom window. Use these markers to zoom in on the area(s) you wish to change. Each parsed injection is numbered with an injection label. The width and position of the injection window can be moved around the injection point as required. Also visible are the nodes used to calculate the position of the baseline.

To change the position of the node, position the mouse pointer over the node, press and hold the left mouse button, and move the node to the desired location. You can also add or delete nodes using the add or delete nodes icons on the toolbar.

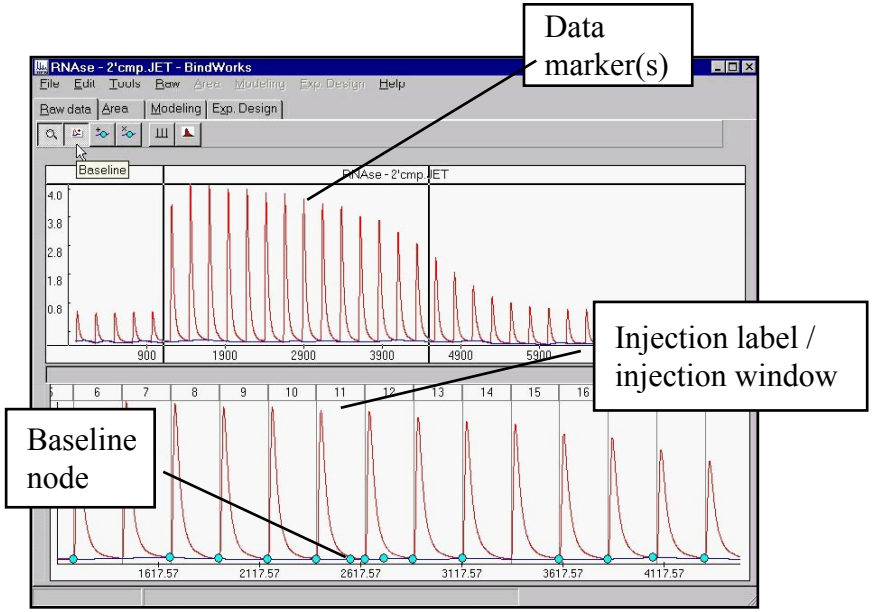

Figure 5-21: The baseline, baseline nodes, and data markers.

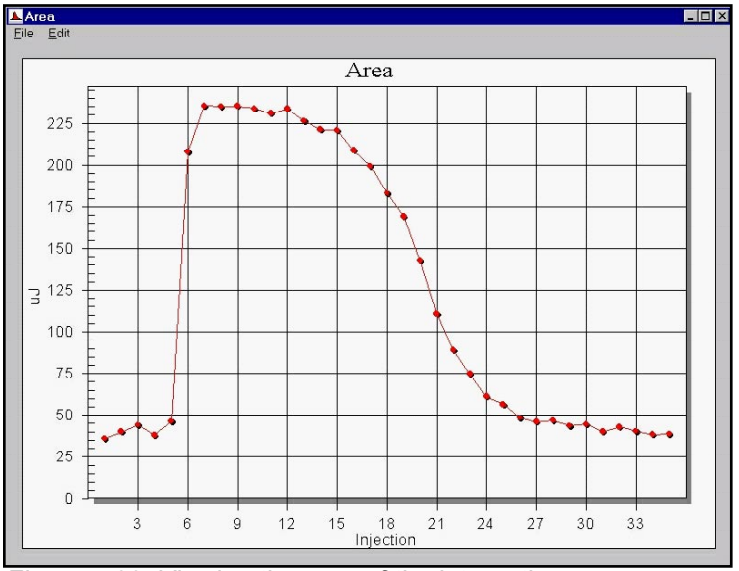

Figure 5-22: Viewing the area of the integration.

As you change the baseline or the position of the injection window relative to the injection, the integration area of the peak will change accordingly. To view the overall affect of your change on the integrated peaks, click the Area icon. A graph of the integrated area will appear (Figure 5-22).

When you have completed the changes you can save your changes to the raw data file by using the *File⇒Save* or *File⇒ Save As* commands.

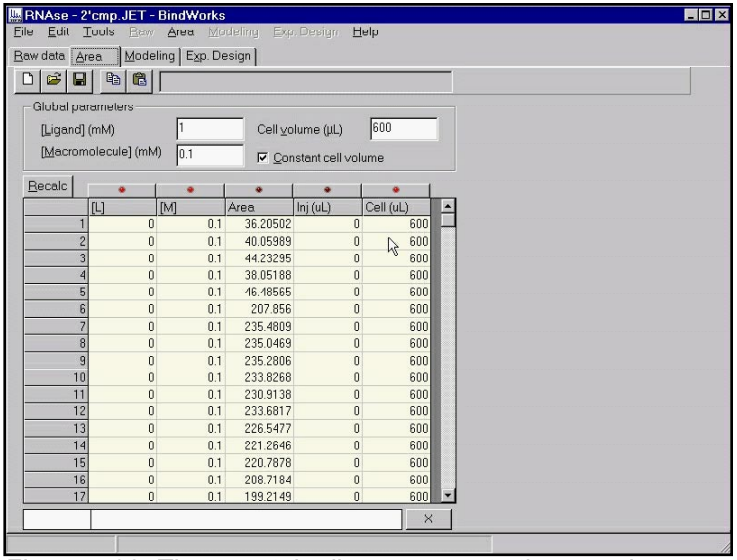

Figure 5-23: The area tab allows access to the experiment spreadsheet.

Integration of the peak

The Area tab allows you to enter or change parameters critical to proper analysis of data prior to final integration. Area calculations are based on the data previously loaded under the Raw Data tab. If you entered the key information required for integration when you set up the experiment, few, if any, changes are required. Most of the data you entered will be imported to BindWorks.

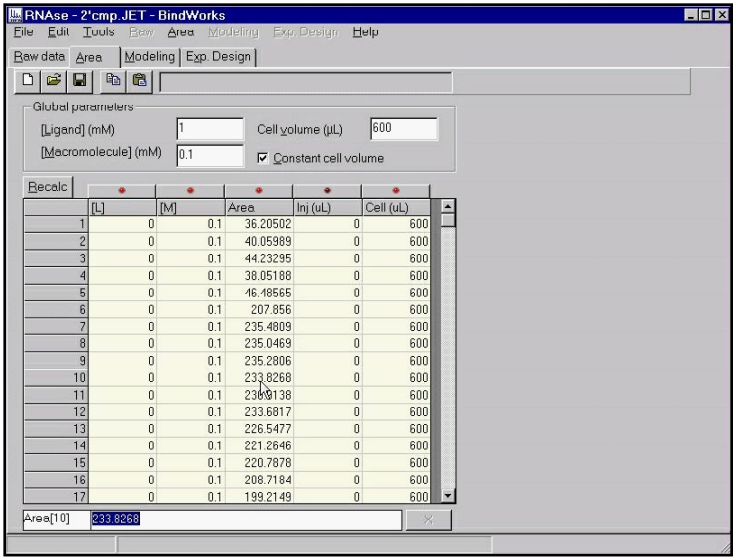

Figure 5-24: Changing the value of an injection.

The initial Area display is shown in Figure 5-23. If you did not enter values for Ligand, Macromolecule, and cell volume when you ran your experiment, enter the values in the appropriate box.

If, for some reason, a parameter of a particular injection (or injections) changed, you can revise the value in the data spreadsheet. To change a value, highlight it, then enter the new value in the data entry box (Figure 5-24). To recalculate the spreadsheet values, single-click the Recalc button.

When you are satisfied with any additions or changes, select *File⇒Save* to write the changes to disk.

Modeling and Fit of the Data

Once you have checked your raw experiment data for errors and entered the key experiment parameters, you can model and fit the data (Figure 5-25).

To fit and model the data:

1. Select the desired model. (See *Modeling*, for additional information on modeling.)
2. Single-click the Fit Data icon on the toolbar.
3. The computer will calculate the values n, K, and ΔH.

After fitting the data you can test the quality of your fit using BindWorks built-in statistical functions.

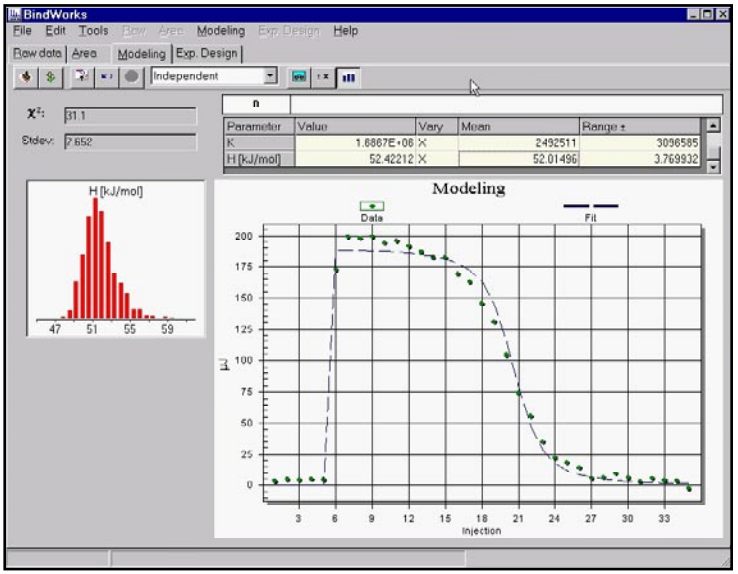

Figure 5-25: A completed model and statistical histogram.

Modeling

Modeling Overview

Each model contains a set of variable parameters. Curve fitting produces optimum values for these parameters. These values will best represent the given input data set.

In addition to user-defined binding models, BindWorks supports three intrinsic binding models:

1. Independent Set of Multiple Binding Sites.
2. Two Sets of Multiple Binding Sites.
3. Cooperative Model.

Intrinsic models are “hard-wired” in BindWorks and cannot be changed or replaced.

Independent Set of Multiple Binding Sites

This is the most common model for a binding experiment. The analytical solution for the total heat is determined by the formula:

$$Q = V \cdot \Delta H \cdot \left[[L] + \frac{1 + [M] \cdot n \cdot K - \sqrt{(1 + [M] \cdot n \cdot K - [L] \cdot K)^2 + 4 \cdot K \cdot [L]}}{2 \cdot K} \right]$$

Equation 5-3

Where the variable parameters for the model are:

ΔH - is the enthalpy of binding

K - is the binding constant

n - is the number of binding sites

Other variables include:

V - is the volume of the cell

[L] - is the total Ligand concentration

[M] - is the protein concentration

(For more information see Freire E.; Mayorga O.; Straume M. *Analytical Chemistry, vol. 62, NO 18, 1990.*)

Two Sets of Multiple Binding Sites

This model assumes the macromolecule has many independent binding sites (at least two). Each site can be one of the two types. Each type has its characteristics (binding constant and enthalpy). The reaction pathways are shown in Figure 5-26.

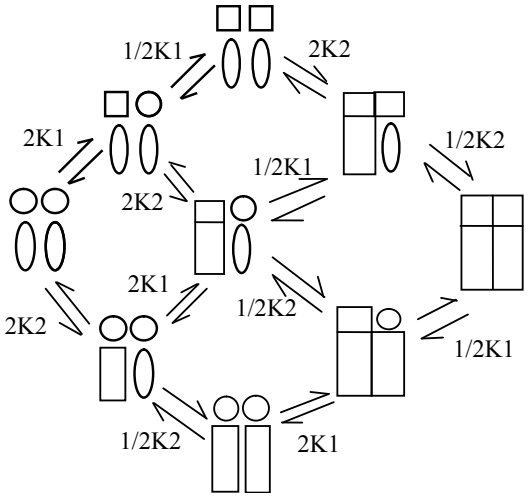

Figure 5-26: Reaction pathways for multiple independent binding sites.

Rectangular regions represent the subunits to which the Ligand is bound. Closed-form equations for this model are as follows:

$$Q = Q_1 + Q_2 = (\Delta H_1[L_{B,1}] + \Delta H_2[L_{B,2}]) \cdot V$$

$$Q = V[M]\left(\frac{n_1 \Delta H_1 K_1[L]}{1 + K_1[L]} + \frac{n_2 \Delta H_2 K_2[L]}{1 + K_2[L]}\right)$$

$$[L_B] = [M]\left(\frac{n_1 \Delta H_1 K_1[L]}{1 + K_1[L]} + \frac{n_2 \Delta H_2 K_2[L]}{1 + K_2[L]}\right)$$

$$[L] = [L_T] - [M]\left(\frac{n_1 K_1[L](1 + K_2[L]) + n_2 K_2[L](1 + K_1[L])}{(1 + K_1[L])(1 + K_2[L])}\right)$$

$$[L] = 2(-y)^{1/2} \cos\left(\frac{\phi}{3}\right) - \frac{B}{3A}$$

$$\phi = \arccos\left(\frac{-x}{(-y^3)^{1/2}}\right)$$

$$x = \left(\frac{B}{3A}\right)^3 - \frac{BC}{6A^2} + \frac{D}{2A}$$

$$y = \frac{C}{3A} - \left(\frac{B}{3A}\right)$$

$$A = K_1 K_2$$

$$B = K_1 + K_2 + K_1 K_2 [[M](n_1 + n_2) - [L_T]]$$

$$C = 1 + [M](n_1 K_1 + n_2 K_2) - [L_Y](K_1 + K_2)$$

$$D = -[L_T]$$

Figure 5-27: Equations for a multiple independent binding site model.

(For more information see Freire E.; Mayorga O.; Straume M. *Analytical Chemistry, vol. 62, NO 18, 1990.*)

Cooperative Binding Model

This model assumes at least two binding sites on the macromolecule as well as interaction between the two ligand molecules (Figure 5-28). For two binding sites the variables that define the cooperative binding are 2 sets of ΔH and β. The first set defines interaction between the macromolecule and the first ligand molecule. The second set determines the interaction between the macromolecule and second ligand molecule. The third one defines the interaction between the two ligand molecules.

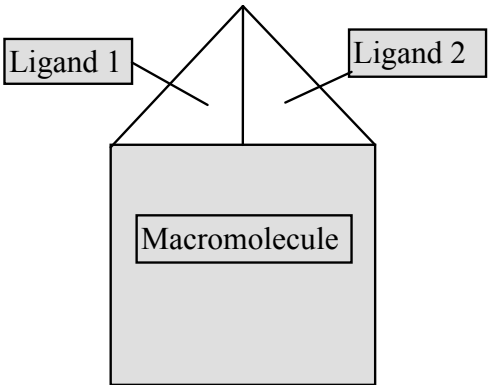

Figure 5-28: Cooperative Binding.

In cases when the number of binding sites is equal to three, number of sets will be equal to six, etc., BindWorks includes implementations for the cooperative model with two and three binding sites as a separate models Cooperative (2 sites) and Cooperative (3 sites). The general Partition function for the cooperative model:

$$Z = 1 + \sum_{n} \beta_i x^i$$

Equation 5-4

Where β is Adair's equilibrium constants and x is the free ligand concentration. The degree of saturation can be expressed as:

$$\overline{Y} = \frac{\partial \ln Z}{\partial \ln X} = \frac{1}{Z} \sum_{n} i\beta_i x^i$$

Equation 5-5

The total amount of ligand:

$$X_t = X + X_b$$

Equation 5-6

Where X_b is the amount of ligand bound per unit volume. Free ligand concentration can be obtained by solving:

$$X_t - X - M \cdot \overline{Y} = 0$$

Equation 5-7

Where M is concentration of macromolecule. The cumulative heat effect is:

$$Q = \frac{Vcell}{Z} \sum_i \beta_i X^i \Delta H_i$$

Equation 5-8

(For more details refer to J. Wyman, S. J. Gill *Binding and Linkage*. University Science Books 1990.)

Designing an Experiment

A calorimetric binding experiment can be designed to measure only the enthalpy change, ΔH, and number of binding sites, n, or to measure the binding constant, K, in addition to ΔH and n. In the first type of experiment, known as a stoichiometric binding experiment, all of the injected ligand is bound to the protein until all of the binding sites are filled. Consequently, all of the initial injections result in the same integrated heat until saturation is reached. Subsequent injections then yield only the heat of dilution of the ligand.

In an experiment designed to measure K, there is curvature to the plot of the heat versus the number of injections. It is the fitting of this curvature that permits a determination of K. In designing an experiment we want to identify conditions in which we expect to observe this curvature. In order to achieve the curvature in the titration experiment necessary to determine K, the product of K and the protein concentration, C_{prot}, must be between 10 and 1000 (*see* Wiseman, T., Williston, S., Brandts, J.F. and Lin, L.-N. (1989) *Anal. Biochem.* **179,** 131). If this product is too high, there is no curvature in the plot. If this constant is too low, then the curvature is too gradual to allow a good determination of K. Consequently, very low concentrations of protein are required to measure very tight affinity constants.

In an experiment designed to measure K, the need to keep the product of K and C_{prot} near 100 must be balanced against the need for a detectable heat. For this reason, very tight binding constants cannot be determined directly unless ΔH is very large so that very low concentrations of protein can be used. Competition experiments provide an alternative means of determining tight binding energetics.

Appropriate experimental conditions for single site binding systems can be determined with estimates of K and ΔH using the Experiment Design tab in the BindWorks program. Enter the parameters as shown in Figure 5-29.

Note that for this experiment, in which a total of 200 µL of titrant will be added to the initial 1300 µL of titrate, a ratio of 10:1 is selected for the titrant to titrate concentration. This will give equal moles of protein and ligand after 12-13 injections so that sufficient excess ligand can be added to give a post-saturation baseline.

A plot of the cumulative heat versus the injection number is shown to the right of input values. Note that there is a sharp transition between the rising portion of the curve and the plateau towards the end of the titration. This sharp transition indicates that K could not be determined from these data.

While K cannot be determined from this type of experiment, the value of ΔH can be determined even if the concentration of protein in the cell is uncertain because all of the injected ligand binds. The heat generated in each of the initial injections is the product of ΔH, the injection volume, V_{inj}, and the concentration of ligand being injected, C_{inj}.

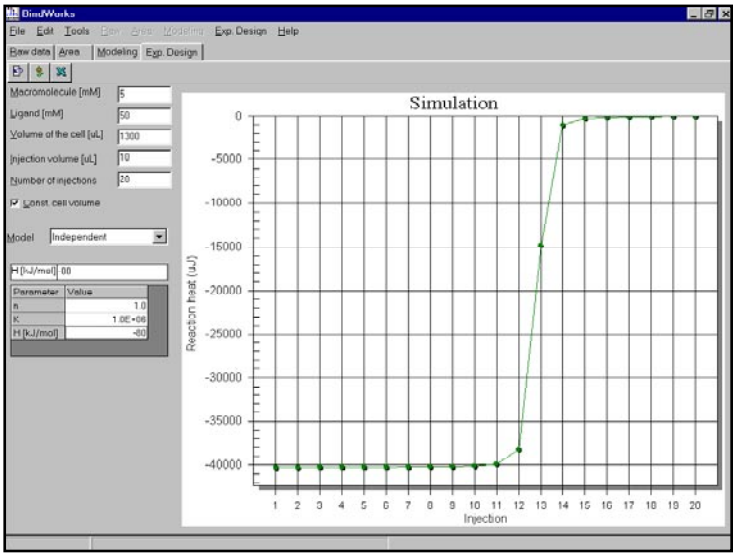

Figure 5-29: The BindWorks design tab. The image shown illustrates a poorly designed experiment.

Enter 1 mM for the ligand concentration and 0.1 mM for the macromolecule concentration. This gives a product of K and C_{prot} of 100 (using *molar* concentration value of the protein). The resulting data now show excellent curvature and significant heat effects indicating ideal experimental conditions. (See Figure 5-30).

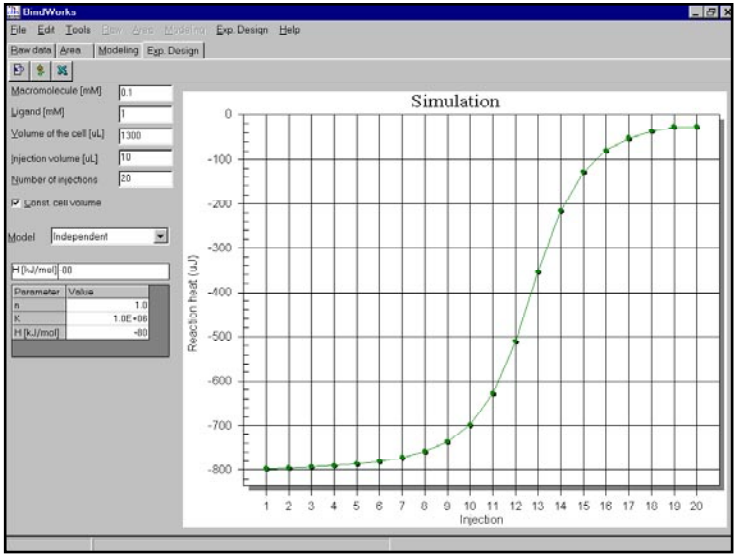

Figure 5-30: BindWorks Experiment Design Tab — Illustration of a well designed experiment.

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Chapter Six: Sample Experiments

Experiment Overview

Following is a brief description of the main steps of a titration experiment. The next segment, Experiment Walk-Through, provides a more detailed presentation of a typical experiment from start to finish. Read through these segments completely before performing any of the operations described.

1. Load the sample cell with titrate and the reference cell with the appropriate buffer or water. While ITCRun is idling, the N-ITC III cells must be allowed to equilibrate so that a stable baseline can be observed; i.e., the heat reading on the calorimeter does not exhibit prolonged upward or downward trends (refer to Calorimeter Specifications, Chapter 1: Introduction).

2. Load a syringe with the titrant, making sure to remove any bubbles, but keep a small air gap (5-10 µL) between the plunger and the titrant solution. Wipe the needle with a tissue and then load the syringe into the burette handle. Install the burette/syringe assembly in the N-ITC III and start the stirrer (150-200 rpm). Allow the system to re-equilibrate until the calorimeter heat reading is stable. Allowing the loaded syringe to remain in the cell much more than 15 minutes before starting the experiment will result in elevated baseline heat caused by diffusion of titrant into the cell, as well as a smaller titration peak for the first injection.

3. When a stable baseline is evident, click on *Experiment⇒Start* or the Go icon at the top of the toolbar and the experiment will begin. Enter a file name when prompted. The program indicator on the Setup tab will be red when the program is active. You will be able to watch the progress of the experiment under the Monitor tab or the Data tab.

4. When the experiment is complete, proceed to data analysis in BindWorks (see Chapter 5: Software Description).

Experiment Walk-Through

Introduction

This chapter contains descriptions of two well characterized titration experiments which utilize materials readily obtainable and provide new users with the opportunity to develop techniques and skills essential for the effective use of the N-ITC III. The first experiment, heat of protonation of Tris base may also be used as a chemical calibration to verify performance of the calorimeter and its settings. The second experiment, binding of 2'-CMP to RNase A, is included as an example of a typical well designed titration experiment.

Chemical Calibration

A chemical calibration tests all aspects of the instrument including the calibration constant, the cell volume and the injection volume. There are several standard reactions which are often used in calibrating isothermal titration calorimeters (see Briggner, L.-E. and Wadsö, I. (1991) Test and Calibration Processes for Microcalorimeters, with special reference to heat conduction instruments used with aqueous systems *J. Biochem. Biophys. Methods* **22,** 101-118.). Here we will describe one: protonation of Tris base (Tris(Hydroxymethyl) Aminomethane). The Tris protonation experiment may be used to determine or verify the calibration factor value setting used in the ITCRun software.

Heat of Protonation of Tris Base

Sample Preparation

Prepare a solution of Tris base by dissolving approximately 3 g in 100 mL of distilled water. The solution will be approximately 250 mM, but the exact concentration is not important so long as it is well in excess.

A 1.00 mM HCl solution is most readily prepared by pipetting 10 mL of standardized 0.1 N HCl into distilled water and diluting to 1 L in a volumetric flask. Alternatively, a standard solution of HCl can be purchased commercially or standardized by acid-base titration (*see* Skoog, D.A. and West, D.M. (1980) *Analytical Chemistry* (Saunders

College Publishing, Philadelphia), p. 228 ff).

Experiment Setup

Experiment parameters:

Equilibration time	200 s
Time between injections	200 s
Injection size	5 µL
Number of injections	20

Rinse the calorimeter cell three times with Tris solution and then load the cell (Figure 6-1). The reference cell may be filled with degassed water. Allow the cells to thermally equilibrate until the heat reading on the calorimeter is stable.

Load the 100 µL syringe with the 1.00 mM HCl solution, making sure to remove any bubbles from the syringe. Wipe the needle with a tissue and then screw the syringe completely into the burette drive as shown in Figure 6-2. (Note: Before inserting the syringe into the burette drive, verify that the plunger indicator on the graduated handle is in the fully raised position. Otherwise, mount the burette on the N-ITC III without a syringe

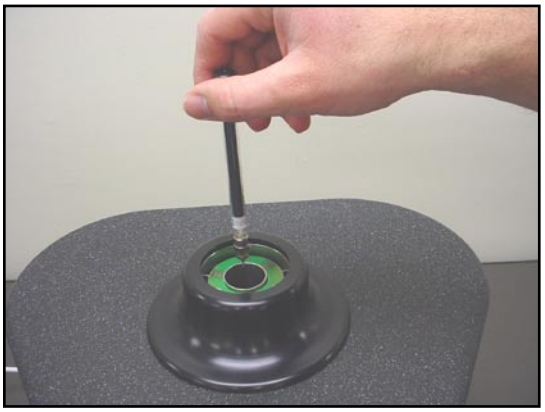

Figure 6-1: Filling the N-ITC III cell.

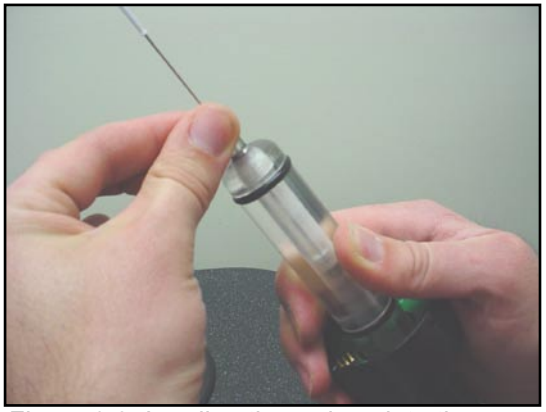

Figure 6-2: Loading the syringe into the burette drive.

Figure 6-3: Inserting the burette drive into the N-ITC III.

and click the burette up icon to initialize the plunger position.) Insert the syringe and burette drive into the N-ITC III as shown in Figure 6-3.

Turn on the stirrer at 150 rpm, allow the system to re-equilibrate until the heat reading on the calorimeter is stable, and then begin the experiment. Enter a file name for the data at the prompt.

Results

The results should be similar to those shown in Figure 6-4. Note that each peak has the same area except for the first. Typically the first injection shows less heat than expected. This is often due to diffusion across the tip of the needle or to difficulties in positioning the burette drive. For 5 µL injections of 1.00 mM HCl at 25°C, the expected heat is 237 µJ. The protonation enthalpy in J/mol at any temperature between 5 and 50°C is given as:

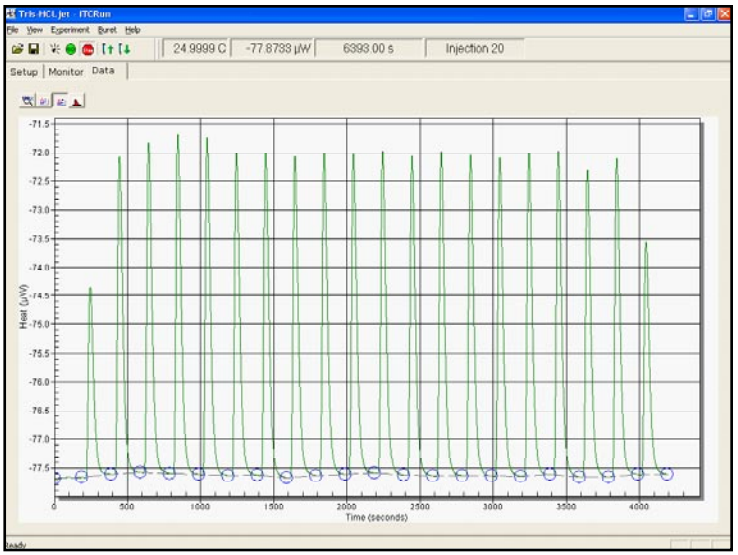

Figure 6-4: Typical calibration graph – Protonation of Tris base. The last injection peak in the figure is smaller because the titrant in the syringe was consumed.

$$\Delta H_{protonation} = -49659 + 102.28t - 0.59275t^2$$

Equation 6-1

where t is given in °C†.

† Christensen, J.J., Hansen, L.D. and Izatt, R.M. (1976) *Handbook of Proton Ionization Heats and Related Thermodynamic Quantities* (John Wiley and Sons, New York).

Sample Experiment

Binding of 2'-CMP to RNase A

Sample Preparation

Prepare 4 L of 15 mM acetate buffer (pH 5.5) by dissolving 5.89 g of potassium acetate, $KC_2H_3O_2$ (FW 98.14), in 4 L of distilled water and adjusting the pH using an HCl solution. Prepare 5 mL of RNase A (FW 13690) solution at approximately 0.07 mM by dissolving 4.8 mg in 5 mL of the acetate buffer. The protein solution should be dialyzed overnight in a refrigerator against the 4 L of acetate buffer using 3500 molecular weight cut-off dialysis tubing.

The 2'-CMP (FW 323.2) solution should be prepared the following day using the dialysis buffer. Remove a sample of buffer (~50-100 mL) and allow it to come to room temperature. Dissolve 8.4 mg of 2'-CMP in 20 mL of buffer for an approximate concentration of 1.3 mM.

The precise concentrations of both solutions must be determined spectrophotometrically. The RNase A concentration should be determined using an extinction coefficient of 9800 cm^{-1} M^{-1} at 280 nm (Wiseman, T., Williston, S., Brandts, J.F. and Lin, L.-N. (1989) *Anal. Biochem.* **179,** 131). The 2'-CMP concentration must be determined at pH 7 by diluting 1 mL of sample up to 25 mL using 100 mM phosphate buffer (pH 7.0). The extinction coefficient is then 7400 cm^{-1} M^{-1} at 260 nm (*ibid.*).

It may be preferable to prepare the above solutions at higher concentrations initially, determine the concentrations spectrophotometrically as described, and then use the dialysis buffer to prepare dilutions at the test concentrations of 0.07 mM for RNase A and 1.3 mM for 2'-CMP. Excess concentrated solutions and an ample amount of buffer may be stored for later use by freezing.

The presence of bubbles in the solutions can result in noisy baselines or spikes during an experiment. It is therefore necessary to remove dissolved gasses from the solutions. Sample degassing is most important for experiments being run at or above ambient temperature since gasses become less soluble with increasing temperature. Degassing is more efficient if the solution is being stirred, but stirring may not be practical for small volumes of solution. Degas 2 to 3 mL of 2'-CMP solution as above, and allow the solution to return to ambient temperature.

Experiment Setup

Experiment parameters:

Equilibration time	200 s
Time between injections	200 s
Injection size	5 µL
Number of injections	20

Rinse the calorimeter cell three times with buffer solution and then load the sample cell with degassed RNase A solution. The reference cell may be filled with degassed water. Allow the cells to thermally equilibrate until the heat reading on the calorimeter is stable.

Load the 100 µL syringe with the 1.3 mM 2'-CMP solution, making sure to remove any bubbles from the syringe. Wipe the needle with a tissue and then screw the syringe completely into the burette drive. (Note: Before inserting the syringe into the burette drive, verify that the plunger indicator on the graduated handle is in the fully raised position. Otherwise, mount the burette on the N-ITC III without a syringe and click the burette up icon to initialize the plunger position.) Insert the syringe and burette drive into the N-ITC III.

Turn on the stirrer at 150 rpm, allow the system to re-equilibrate until the heat reading on the calorimeter is stable, and then begin the experiment. Enter a file name for the data at the prompt.

Blank Titration

The heat effect measured in a titration experiment includes the heat of dilution of the titrant, in addition to the heat generated from the binding reaction. In order to correct for the dilution heat, a blank experiment is performed which is identical to the binding experiment, but with buffer only present in the cell.

Set up a titration control file with twenty 5 µL injections using a 200 s equilibration time and 200 s between injections (parameters should be identical to the sample experiment). Load the syringe with 2'-CMP. When the calorimeter has re-equilibrated, begin a titration.

Results

The graph will appear similar to that shown in Figure 6-5. The data generated in a titration experiment takes the form of power (µW) as a function of time (seconds). In order to analyze the data we want to know heat as a function of the number of injections (or moles of added ligand). This form of the data is obtained by integrating the peaks from the experiment.

To begin, start the data analysis program BindWorks. Next, select *File⇒Open* from the main menu, then select the data file you wish to analyze. Select the **Raw Data** tab from the program main screen and click the **Show Zoom Window** and **Baseline** buttons (Figure 6-6). Adjust the default base lines used to integrate the data if required.

After integration is complete, select the **Area** tab from the

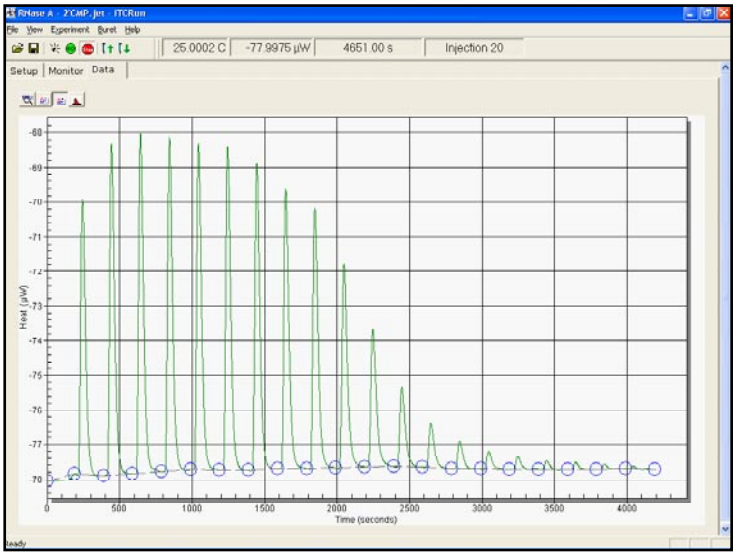

Figure 6-5: Typical data output from RNase A titration experiment.

program main screen (Figure 6-7). Enter the ligand and macromolecule concentrations (based on the spectroscopic determination) and the cell volume. Check the **Constant Cell Volume** box. To subtract the blank run, click on the Area label at the top of the third table column to highlight the entire column, then type in an equal sign followed by a minus sign (=-) followed by the average blank injection area, then click the execute button to the right of the edit box. This will subtract the average blank injection area from all peak areas. After you have made all necessary entries, click the **Recalc** button.

Next, select the **Modeling** tab from the program main screen (Figure 6-8). Select the **Independent** model at the top of the tab. You are likely to notice that the integrated heat for the first injection is smaller in magnitude that would be expected by comparison to subsequent injections. This is caused by diffusion across

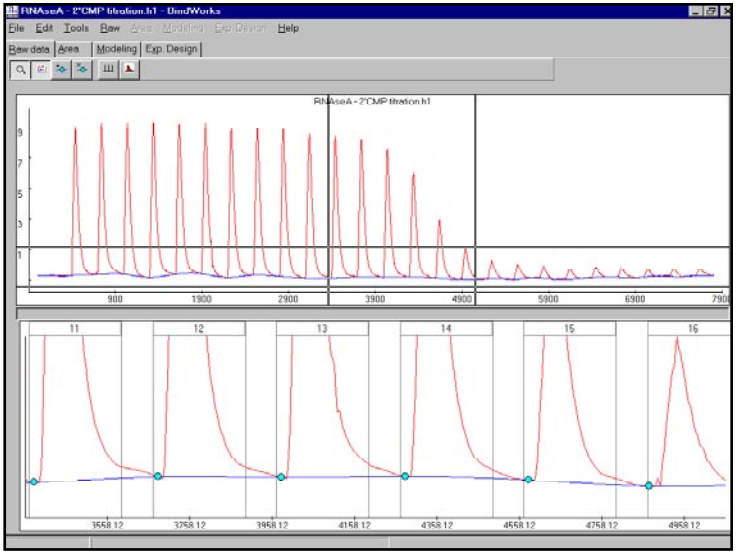

Figure 6-6: Adjusting the baseline for the RNase A experiment.

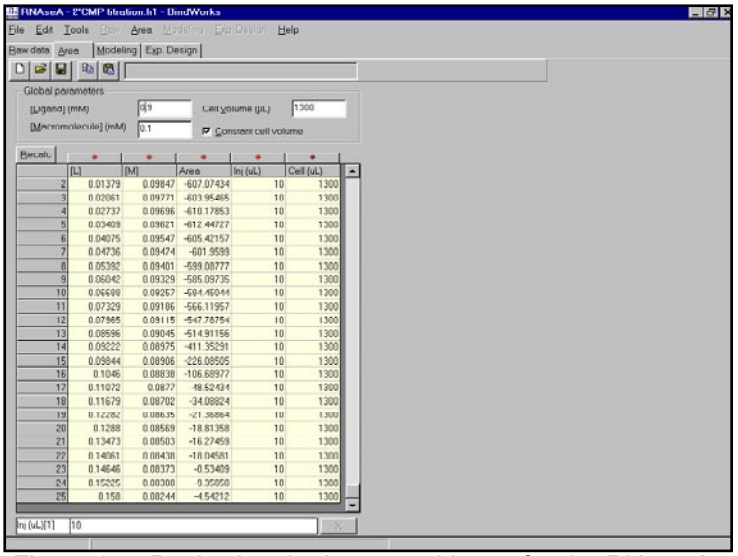
File Edit Tools Raw Area Modeling Exp Design Help
Raw data Area Modeling Exp Design
Global parameters
Constant cell volume
Recalc

Figure 6-7: Reviewing the integrated heats for the RNase A experiment.

the tip of the injection needle during equilibration, which results in fewer moles of titrant being injected in the first injection. If you want to discard this point in the fit then position the mouse pointer directly over the point and click the left button once. The point should be replaced by a red triangle, indicating that the integrated area will not be used in the fit. Next, click the **Fit Selected Model** button. The values for K, ΔH and n will be displayed in a table located directly above a graph showing both the data and fit. If you would like statistical analysis of the fit click the **Statistical Analysis** button.

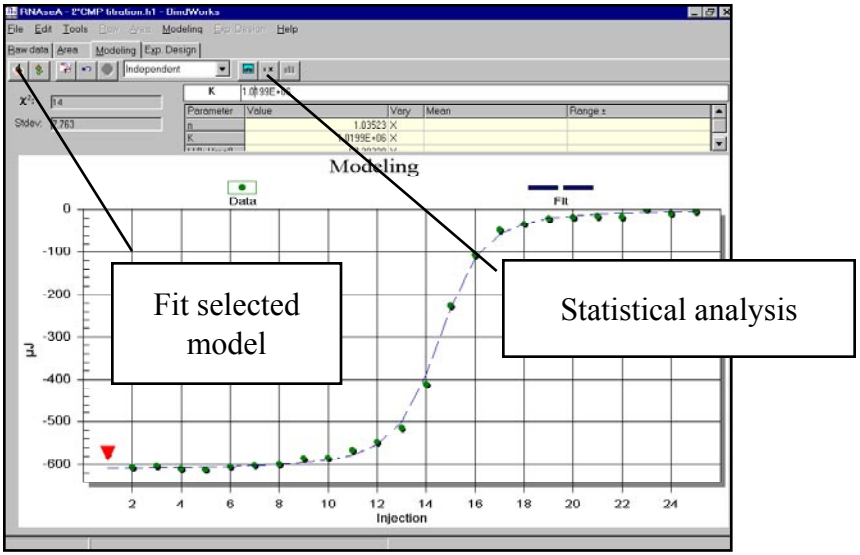

Figure 6-6: Modeling the RNase A experiment.

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Chapter Seven: Troubleshooting

Minimizing Blank Corrections

There will always be blank corrections for experiments. However, minimizing the blank correction can greatly improve experiments. Even when injecting water into water there will be some heat produced due to viscous mixing. The viscous mixing heat is determined by many factors.

There are several steps that can be taken in order to minimize the dilution heats and hence the need for blank titrations. Ligand solution should always be made up in the same dialysis buffer used for the protein. If the ligand is also a protein then it should be dialyzed in the same buffer. Less concentrated solutions also have lower dilution heats and should be used when possible.

Operation Away from Ambient Temperature

The N-ITC III is designed to perform over a wide temperature range. When operating the N-ITC III away from ambient temperature it is important to allow adequate time between injections for the titrant to equilibrate to the calorimeter temperature before injection. Thorough degassing of the titrant is especially important when operating above the ambient temperature.

Stirring Speeds

Adequate stirring is required in order to have rapid mixing of the titrant upon injection, but excessive stirring will result in noisy baselines. Generally, a stirring speed of 150-200 rpm is appropriate.

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